## THE ROLE OF INTESTINAL ALKALINE PHOSPHATASE IN AMINO ACID ABSORPTION IN THE RAT

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UNIVERSITY OF ALBERTA

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Date *16 April, 1957*

The undersigned hereby certify that they have
read and recommend to the School of Graduate Studies
for acceptance, a thesis entitled "The Role of
Intestinal Alkaline Phosphatase in Amino Acid
Absorption in the Rat" submitted by Eugenie
Triantaphyllopoulos, M.D., in partial fulfilment of
the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Alkaline phosphatase levels in the small intestine of the adult male albino rat were found to decrease exponentially from the duodenum to the ileocolic valve. The ten cm. portion of the intestine measured from the pylorus was subjected to cellular fractionation in 0.25M sucrose. Distribution of the activity of the enzyme in various fractions was as follows: nuclei, 7-10%; mitochondria, 6-8%; microsomes, 76%; and the non-particulate fraction, 9%; and the specific activity for these fractions was 3, 10, 52, and 3, respectively. The alkaline phosphatases of the four fractions were indistinguishable on basis of the influence of: time of hydrolysis, pH of the reaction mixture, temperature, enzyme concentration. Only the Michaelis constant and sensitivity to Mg^{++} activation differed slightly for each fraction. Consequently, in subsequent studies unfractionated homogenates in isotonic sucrose were used.

The range of alkaline phosphatase in homogenates of the ten cm. of intestine measured from the pylorus varied in normal rats from 20,000 to 32,000 units/100 gm. of wet tissue. Levels of the enzyme decreased during the first two days of fasting, to values unaltered by further starvation.

After a series of preliminary trials, a method was adopted for force-feeding three-day fasted animals with 0.76M solutions of each of several L-amino acids after adjustments of the pH to about 7. Leucine, arginine, methionine, glycine, glutamic acid, serine, and threonine each produced statistically significant

elevation of intestinal alkaline phosphatase above control
values, while force-feeding of tryptophan was followed by
a lowering of the intestinal level of the enzyme. Only
three of the above L-amino acids affected the activity of
serum alkaline phosphatase. Glycine and glutamic acid
increased levels of the enzyme, while methionine had the
opposite effect, and these results are surprising in view
of the well-known contribution of intestinal tissue to the
serum enzyme levels during absorption of some digestion
products from the gastrointestinal tract. Histidine
affected the values of the serum enzyme only. Solubility
of the amino acid in the gut, as well as speed of absorption,
may account for some of these findings. Yet blood amino
acid concentration was found to be significantly increased
after ingestion of all amino acids except cystine, tryptophan,
or arginine. Ingestion of arginine was followed by a highly
significant decrease in blood amino acid concentration.

Force-feeding of a mixture of glutamic acid, glycine,
and histidine resulted in highly significant elevations of
serum and intestinal alkaline phosphatases, as well as of
the concentrations of amino acids in serum and intestinal
tissue.

A profound depressing effect on intestinal alkaline
phosphatase levels was observed following the force-feeding
of 0.76M solutions adjusted to pH 2 with HCl of each of:
glycine, methionine, lysine, tryptophan, phenylalanine,

histidine, and leucine. Tryptophan solution, adjusted to
pH 11, also decreased the activity of the enzyme. Acid
solutions of glutamic acid were less effective, while
tyrosine was ineffective under these conditions. An
exponential relationship between the milliequivalents of
HCl acid given to each animal and the inhibition of
intestinal alkaline phosphatase was observed.

It would appear from this investigation that intestinal
alkaline phosphatase may participate in the absorption of
some L-amino acids, and that the pH of the solution fed,
especially if strongly acid, has a very profound effect
on the enzyme in vivo.

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THE UNIVERSITY OF ALBERTA

THE ROLE OF INTESTINAL ALKALINE PHOSPHATASE

IN AMINO ACID ABSORPTION IN THE RAT

A DISSERTATION

SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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GENERAL INTRODUCTION

The name "alkaline" phosphatase has been given to enzymes of broad specificity, which hydrolyse monoesters of orthophosphoric acid at an optimum pH around 9; the precise value depending on the source of the enzyme, type of substrate, substrate and magnesium ion concentration, presence of other activators, etc.

The enzyme was first discovered by Grosser and Husler (1). It is widely distributed in all animal cells with the exception of the hyaline cartilage and the tunics of the blood vessels (2). Certain tissues are particularly rich: intestinal mucosa, kidney cortex, bones of growing animals, lactating mammary gland, and to a smaller extent, liver and brain. White blood cells and lymphoid tissues come next. Plant cells contain little or more commonly no alkaline phosphatase (8).

Several attempts have been made to isolate the enzyme. Schmidt and Thannhauser (3) in 1943 obtained a preparation of considerable purity from calf intestinal mucosa. It contained about 21% of polysaccharide, 10.1% N, and had a specific activity of 4,000 units/mgm. of total N. (1 unit =0.1 mgm. of inorganic phosphorus liberated from Na-β-glycerophosphate within 15 minutes of incubation at 37°C and pH 9.3, hydrolysis not exceeding 5% of substrate). Morton's preparation (4) was a colorless, unconjugated protein, substantially free of organic phosphorus and nucleic acid, which contained small amounts of carbohydrate. Tyrosine and tryptophan were present in amounts of

26 and 11 moles/10^5 gm. respectively. Mathies (5) purified
swine kidney phosphatase with a specific activity up to
10,000 units/mgm. of protein N (1 unit=1 mgm. of phenol
liberated from disodium phenyl phosphate in 15 minutes at 25°C).
The electrophoretic and ultracentrifugal pattern revealed it to
be 80-90% of one component. Spectroscopically it was shown to
contain Zn, Mg, Cu and Fe (in order of increasing concentration)
as major components, and Mn, Pb, and P in minor quantities.

The synthetic action of unpurified phosphatase (both acid
and alkaline) from various organs, was observed relatively
early (6,7) and found to be so regular, that the synthesis of
glycerophosphates from glycerol and phosphate by the kidney
enzyme had been chosen in several European manuals as an
example for the demonstration of the synthetic activity of
enzymes in laboratory courses (8). However, very little
attention was given to the biological significance of this
action until recently. Meyerhof and Green (9) in 1949 obtained
the synthesis of the phosphate esters of all the natural sugars
from inorganic phosphate and chemically pure sugar, in the
presence of purified intestinal alkaline phosphatase. A year
earlier Axelrod (10,11) had reported, that a direct transfer of
phosphate occurred from some aryl phosphates to certain
aliphatic alcohols, in the presence of citrus phosphatase, (acid)
without phosphate passing through the inorganic state. Meyerhof
and Green (12,13) reported the occurrence of transphosphorylation
reactions from organic phosphate compounds to common biological
polyalcohols in the presence of purified intestinal alkaline
phosphatase. In the beginning, they thought that the transfer

was always from a higher to a lower level of energy and that the

velocity of the reaction was proportional to the energy

difference between phosphate donor and phosphorylated product.

But later (14) as a result of new experiments, they revised

their views. They reported that "the difference in the rates

of phosphorylation to glycerol could not be explained by the

energy content of the phosphate donor alone, rather it seems

that the relative affinities of the phosphate donors for the

enzyme and the activity of the resulting substrate-enzyme

complex play an important role in the speed factor."

Morton's experiment (15) with electrophoretically

homogeneous preparations gave further proof to the transphos-

phorylating properties of the non-specific phosphatases.

Various physiological processes are related to phosphatase

activity: bone calcification, resorption of sugar from the

convoluted tubules of the kidney, absorption of some foodstuffs

from the intestine (16-27), and protein formation in the cells.

It was possible to demonstrate the appearance and evolution of

phosphatase in: the course of cellular differentiation during

the embryonic development of the chick and of mammals, the repair

of wounds, cutaneous burns, and bone fractures, and the formation

of secretions of protein nature, such as silk (8). Cytochemical

studies have shown that alkaline phosphatase exists

characteristically in chromosomes, newly formed collagen and at

the secretory border of a wide variety of secretory cells.

Danielli (28) suggests, that many of the phosphatases are

integral parts of contractile proteins and that for the most part the enzyme acts not as a phosphatase, but as a phospho-kinase, transferring a phosphate group from a higher to a lower energy bonding, so that the difference in energy between the two compounds would be available for the protein contraction.

Increased serum alkaline phosphatase activity has been associated with skeletal diseases (29) (rickets, Paget's disease, osteogenic sarcoma) and liver disorders (obstructive and hepatocellular jaundice, portal cirrhosis etc.). Decrease in blood phosphatase has been observed in scurvy and hypothyroidism.

Adrenocorticotrophic hormone (30) has been reported to decrease the plasma alkaline phosphatase of the rat, while the adrenocortical hormones (31,107) showed the opposite effect on intestinal,liver,lung, spleen and thymus phosphatases.

The increase of blood alkaline phosphatase after ingestion of certain foodstuffs (17-19,22,32-35) was shown to be intestinal in origin (18,19,22). Hence attention was directed to the intestinal counterpart of the enzyme. Considerable research has been done in this laboratory concerning this subject. It was shown by Tuba and Robinson (24), Tuba and Dickie (25,26), and Dickie et al. (27), that a significant rise in intestinal alkaline phosphatase activity above fasting levels was produced after the feeding of olive oil, glucose, galactose, fructose, mannose, casein, vitellin, as well as by butyric, lauric and palmitic acids. These authors concluded that intestinal alkaline phosphatase in the albino rat participates in the absorption of some fatty acids, carbohydrates, and proteins.

The present study was undertaken as an extension of
their work in order to investigate the effect, if any, of
ingestion of pure amino acids on the intestinal alkaline
phosphatase.

In a complicated system, such as the animal organism,
tissues and organs are not isolated nor independent from
each other. Changes in one will often affect others.
Blood, circulating through every part of the body, is usually
the first to reflect such changes. For this reason, it was
thought profitable to extend this investigation to include
the blood enzyme also. Determinations of blood and intestinal
alkaline phosphatases, carried along at the same time, would
give a better understanding of the amino acids absorption
mechanisms than determinations of intestinal phosphatase alone.

EXPERIMENTAL

(a) The experimental animal

Adult male albino rats which weighed approximately
270-350 gm. were used in all experiments. They were housed
in group cages and maintained on Purina fox checkers
ad libitum prior to each experiment. Rats which met the weight
requirements were selected each time. For the study of the
kinetics and the distribution of the enzyme in the intestine,
the animals were killed immediately after selection. Rats
chosen for feeding experiments were housed in individual cages
without food, but with an adequate water supply, for a few
days, before the amino acid under investigation was fed.

All animals were killed by decapitation. Whenever serum
phosphatase and amino acid determinations were necessary, the
blood flowing from the neck was collected and the serum
separated within 2 hours. Samples of 0.5 ml. from each serum
were placed in individual tubes and kept frozen for amino acid
determinations. A second aliquot of each serum was diluted with
demineralized water 1:25 in the case of sera from fasted animals
or 1:50 in the case of sera from normal rats. The diluted sera
were stored in the cold room (at $4^{\circ}C$) in order to be used for the
determination of the phosphatase activity.

For intestinal alkaline phosphatase and amino acid
determinations either the entire small intestine, cut in sections,
or only the first 10 cm. from the pylorus were used according to
the type of experiment. The required portion or portions were
carefully excised, washed out with a stream of cold isotonic

sucrose solution, blotted dry lightly, weighed to the nearest
hundredth of a gram, and finally stored in the freezing
compartment of the refrigerator. The next morning, the tissue
was homogenized with demineralized water (or with isotonic
sucrose for fractionation and kinetics studies) in a Potter-
Elvehjem homogenizer and made up to 25 ml. Two ml. were
immediately pipetted into a test tube and frozen to serve for
amino acid determinations, whenever this was necessary. The
original homogenate was either fractionated in a refrigerated
ultracentrifuge into nuclear, mitochondrial, microsomal and
supernatant fractions or used unfractionated for phosphatase
determinations after the proper dilution.

Phosphatase determinations both in blood and intestine
were always carried out within 48 hours after the death of the
animal (more commonly within 20 hours). The enzyme from both
sources was found to be stable for even longer periods of time.
The tests for amino acids were performed within 2-6 days. The
samples were kept frozen all this time to avoid autolysis which
was fairly rapid (36,37) in the case of intestinal homogenates,
although they were kept in the cold room all the time. It was
found that the α-amino-nitrogen concentration increases after
storage of intestinal homogenates in the cold room by about 300-580% in 14 days.
(b) <u>Methods</u>

Alkaline phosphatase of intestinal homogenates and blood
sera was determined by the micromethod of Shinowara, Jones, and
Reinhart (38) as modified by Gould and Schwachman (39). The
unit of phosphatase was defined by Shinowara as "equivalent to

1 mgm. of phosphorus liberated as phosphate ion during 1 hour
of incubation at 37°C with a substrate containing sodium
β-glycerophosphate, hydrolysis not exceeding 10% of the substrate;
and at optimum pH $9.3\overset{+}{-}0.15$."

Reagents

1. Substrate - buffer solution

  0.4240 gm. of sodium diethyl barbiturate

  0.5000 gm. of β-glycerophosphate (sodium salt)

  0.2464 gm. of magnesium sulfate heptahydrate, dissolved

  in 100 ml. of cold demineralized water.

2. Molybdic acid

  This was prepared immediately before use by adding
  (solution)
  quickly one part of 7.5% sodium molybdate solution to
  one part of cold 10N sulfuric acid and mixing them well.
  It is important that the molybdate is added quickly. On
  slow addition the molybdic acid solution acquires a
  light yellow tinge and the blank becomes blue.

3. Stannous chloride

  I. Stock solution: 6.0 gm. of stannous chloride + 10 ml.
   of concentrated HCl. Covered with a toluene layer
   and stored in the refrigerator.

  II. Dilute solution: 0.1 ml. of stock solution diluted to
   50 ml. with cold demineralized water immediately before
   use.

4. Stock phosphorus solution: 0.4394 gm. of potassium
  diphosphate (KH_2PO_4) made up to 1 litre (100.1 micrograms
  of phosphorus per ml.)
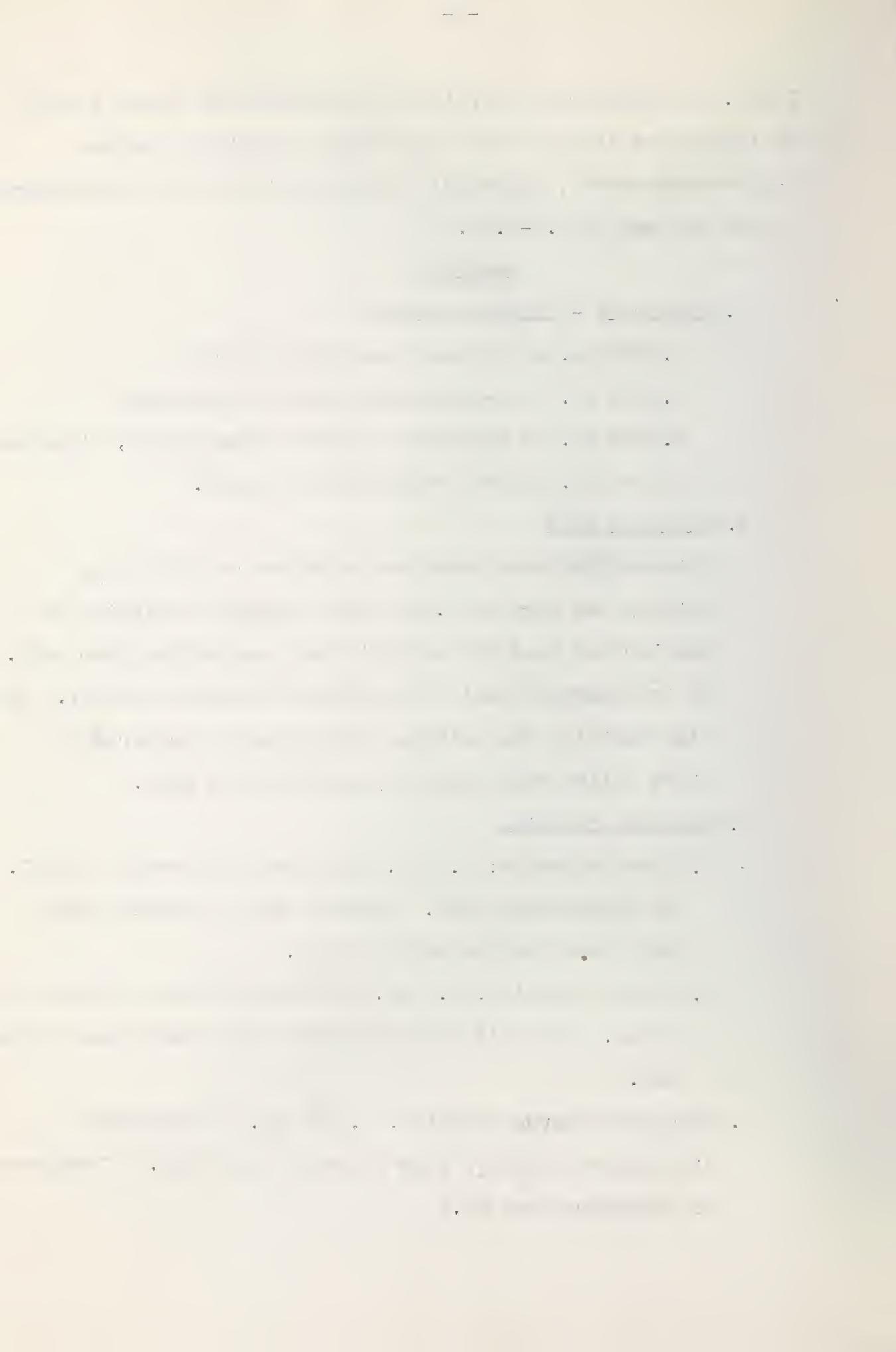
Method

Serum Phosphatase

Diluted serum[*] (0.5 ml.) was pipetted into a 100 x 12 mm. test tube and 0.5 ml. of distilled H_2O into a similar tube (blank). Both tubes were placed in a water bath at $37°C$ until the fluids reached incubator temperature. Then 1.0 ml. of substrate-buffer solution, also at $37°C$, was added to each tube and the reaction was allowed to proceed for exactly 60 minutes, when 1.0 ml. of 10% trichloracetic acid was added to stop the reaction and precipitate the proteins. The tube with the test mixture was centrifuged in an ordinary clinical centrifuge for a few minutes. From each of the tubes, 1.5 ml. of the clear fluid was pipetted into two 14 x 125 mm. test tubes, and to these were added: 2.1 ml. of 0.1N NaOH, 1.2 ml. of molybdic acid and 1.2 ml. of dilute stannous chloride solution. Each tube was shaken thoroughly after the addition of each reagent to insure good mixing. The intensity of the blue color was determined in a Coleman Universal spectrophotometer (Model 14) at 600 mμ, 23 minutes after the addition of the last reagent (40).

The amount of phosphorus contained in the tube was estimated by means of a standard curve. The values for the curve were obtained by plotting the amount of phosphorus in micrograms contained in a number of dilutions of the stock KH_2PO_4 solution, against the optical density of the color obtained by the above procedure. Pre-existing serum inorganic P values were determined in tubes which contained no substrate and these values were

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[*] Serum from fasted animals was diluted 1:25

Serum from normal animals was diluted 1:50

subtracted from the amount of P present in the reaction tube
after one hour of hydrolysis to give the phosphatase activity
in units/100 ml. serum.

Intestinal Phosphatase

The intestinal homogenates were diluted 1:6,250. In this
dilution, the concentration of the protein becomes so small that
for practical purposes it is negligible;   no precipitate was
visible after the addition of the trichloracetic acid. The
method therefore was shortened appreciably: 0.3 ml. of each
diluted homogenate were incubated with 0.6 ml. of substrate-
buffer solution for one hour. The reaction was stopped by the
addition of 2.5 ml. of molybdic acid (diluted 1:2) followed by
2.6 ml. of stannous chloride (0.1 ml. of stock solution made up
to 100 ml. of H_2O). The final volume was 6.0 ml. and the
concentration of each component was the same or about the same
as in the method for serum alkaline phosphatase described above.
The **final pH** of the solution and the intensity of the color were
unaffected by the alterations in technique. Determinations
performed by the modified and the original method gave the same
values.

All determinations were carried out in duplicate and agreed
within 1-3%.

Determination of amino acids

The method of Frame, Russell, and Wilhelmi (41), as modified
by Russell (42) was used. This method depends upon the reaction
of the α-amino group of amino acids with β-naphthoquinone-4-sulfonic
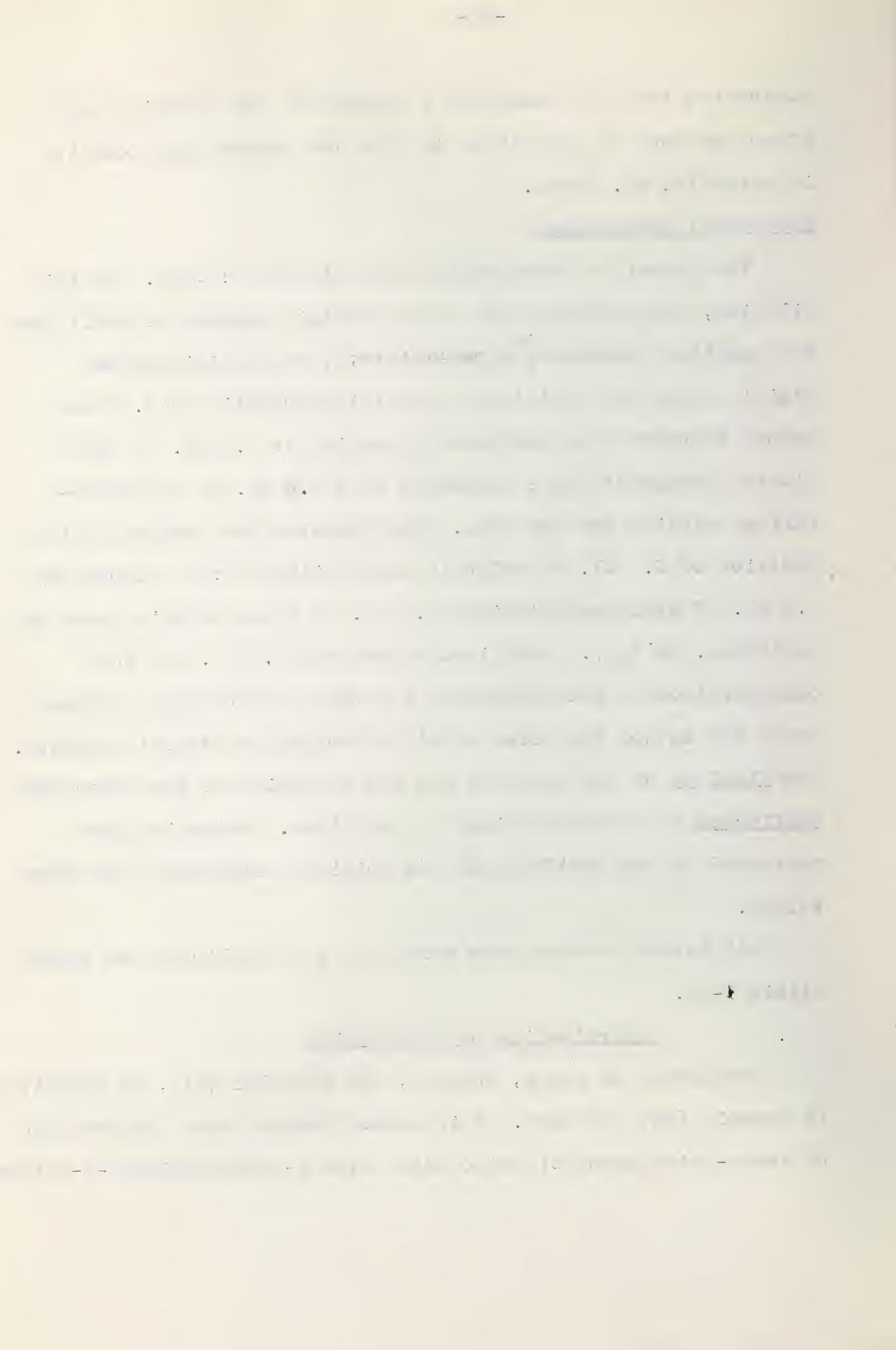
acid. The intensity of the color produced gives a measure of
the concentration of the amino acids.

$$2 \quad \text{(naphthoquinone sulfonate)} + ANH_2 \longrightarrow \text{(naphthoquinone-NR)} + \text{(naphthalenediol)} + SO_2 + H_2O$$
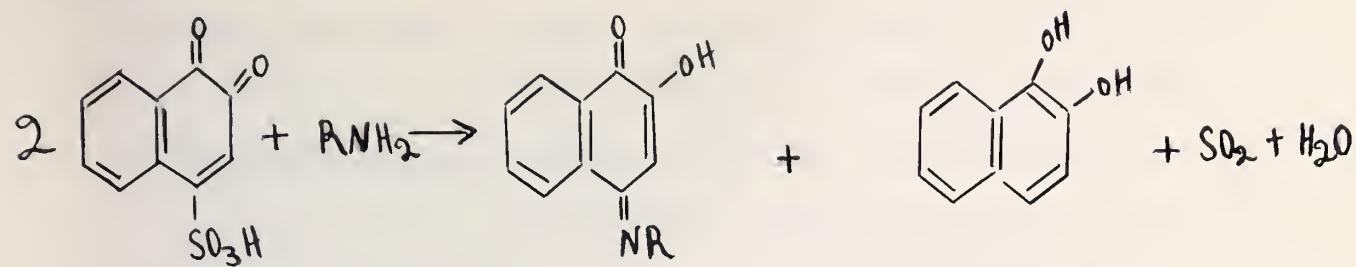
Reagents

1. <u>Amino acid standard solution</u>: a mixed standard containing
   glycine and glutamic acid was used because this produced
   a colour comparable to that produced with serum under the
   same conditions.

   (a) <u>Glycine standard</u>

         0.2680 gm. of pure dry glycine.

         35 ml. of N HCl.

         1 gm. of sodium benzoate.

         made up to 500 ml. with distilled water.

   (b) <u>Glutamic acid standard</u>

         0.5250 gm. of pure dry glutamic acid.

         35 ml. of N HCl.

         1 gm. of sodium benzoate,

         made up to 500 ml. with distilled water.

   Each of these stock standard solutions contains 0.1 mgm.
   of amino acid nitrogen/ml. They are stable indefinitely.

   (c) <u>Mixed standard</u>: 1.5 ml. of each of the above standards
   was pipetted to a 25 ml. volumetric flask and made up
   to volume with distilled water. This standard contained
   0.03 mgm. of amino acid nitrogen/2.5 ml.

2. <u>0.25% alcoholic phenolphthalein</u> (in 95% alcohol).

3. <u>0.1N NaOH</u>.

4. <u>Borax solution</u>:   20 gm. of borax ($Na_2B_4O_7 \cdot 10\ H_2O$) per
   litre of water. Stable indefinitely.

5. <u>Naphthoquinone solution</u>:   0.25 gm. of β-naphthoquinone
   -4-sulfonic acid dissolved in 50 ml.
   of water. It was always prepared
   just before use.

6. <u>Acid formaldehyde solution</u>:   0.30N HCl containing 0.04M
   formaldehyde (3 ml. of 40% formaldehyde
   per litre). Stable indefinitely.

7. <u>0.05M sodium thiosulfate</u>:   (12.42 gm./litre). Need not
   be standardized. Usable indefinitely.

<center>Procedure</center>

The serum was deproteinized by the addition of 5
volumes of 2/9N H_2SO_4, 1 volume of sodium tungstate, and
3 volumes of water. Protein-free filtrates from intestinal
homogenates were prepared in the same way by the addition of
1.25 ml. of 2/9N H_2SO_4, 0.25 ml. of sodium tungstate, and
1.5 ml. of H_2O to 2 ml. of concentrated homogenate. The
precipitate from intestinal samples was very fine and
necessitated the use of Whatman no. 5 filter paper in order
to obtain a clear filtrate.

From each filtrate, aliquots of 2.5 ml. were transferred
to graduated test tubes. Into similar tubes, 2.5 ml. of diluted
amino acid standard and 2.5 ml. of water (blank) were placed

separately. To each tube were added: 1 drop of alcoholic
solution of phenolphthalein, one drop of about 1.2N NaOH
and finally 0.1N NaOH drop by drop until a permanent pink
color was obtained. Aliquots of 0.5 ml. of borax solution
were pipetted next and these were followed by 0.5 ml. of
freshly prepared naphthoquinone solution, mixing well after
each addition. The tubes were placed immediately in a
boiling water bath; after 10 minutes they were transferred
to a cold water bath for 5 minutes. After the volume of
each tube was adjusted to 6.5 ml. with distilled water, 0.5 ml.
of acid formaldehyde and 0.5 ml. of thiosulfate solutions
were added and the contents of each tube were mixed thoroughly.
The colors were read 10-30 minutes after the addition of the
last reagent, in a Coleman Universal Spectrophotometer (Model 14)
at 480 mμ.

Two to four standards were always used for comparison
with each set of determinations. They usually checked within
1-3%.

PART I   DISTRIBUTION AND KINETICS OF INTESTINAL

ALKALINE PHOSPHATASE

INTRODUCTION

Almost all the research done with intestinal alkaline
phosphatase so far, has been concerned mainly with the first
10 centimeters of the small intestine and almost exclusively
with the supernatant fluid of the homogenates. Exceptions
to this are the work of Jackson (23), Lawrie and Yudkin (21)
who used the whole intestine, and Dickie (43) who did some
fractionation studies with the first 10 centimeters.

Dickie found that the activity of the unfractionated
homogenate was lower than the sum of the activities of the
cellular fractions and concluded that some activators or
inhibitors must be present in the fractions, which affected
the activity of the enzyme. It was therefore decided to
investigate further these observations.

Tissue fractionation is a relatively new technique.
Isolation of individual cellular components had been
accomplished as early as 1913 (44) and 1934 (45). However,
it was only during the late nineteen forties that the first
separation of tissue homogenates into nuclear, mitochondrial,
microsomal and soluble fractions was performed by means of
differential centrifugation. Hogeboom, Schneider, and Pallade
published the first satisfactory technique concerning this
subject (46).

Fractionation is usually performed in a refrigerated
centrifuge in order to minimize the autolytic processes, which

are enhanced by the high temperatures developed during

centrifugation.

A good fractionation technique must:

(1) separate the various cellular components into "pure"
   fractions.

(2) preserve the morphology, biochemical properties and
   vitality of these components as long as possible.

In order to reach this goal:

(a) careful and complete homogenization of the biological

material should be assured. For this purpose the Potter-

Elvehjem homogenizer is considered the best (47); it has been

found (48) that practically all the cells are broken, while

the nuclei and other subcellular components remain   intact.

Homogenization with the Waring blendor or mortar and pestle

have two serious disadvantages: (i) too many cells remain

undisrupted (ii) nuclei may be broken. In addition, the

Waring blendor produces excessive frothing and heating.

(b) Selection of a suitable suspension medium (47,49) is

necessary.

It has been noted that mitochondria become swollen, when

they are suspended in distilled water. In isotonic saline

or other salt solutions they are strongly agglutinated and

sediment with the nuclei. Moreover, they lose their property

of being stained vitally with Janus Green B. Isotonic (0.25M)

and hypertonic (0.88M) sucrose solutions have been found to be

the most satisfactory. They preserve the normal size and

vitality of mitochondria. In hypertonic sucrose solutions

many of the mitochondria actually keep their elongated natural

form. Biochemical tests have also been applied in order to check the integrity of the particulate components: distribution of deoxyribonucleic acid to check the disruption of nuclei, and determination of succinoxidase activity in the absence of added cytochrome c. Isotonic sucrose solutions are usually preferred because lower gravitational forces are required than when hypertonic sucrose solutions are used.

(c) Selection of the proper speed and duration of centrifugation. The most commonly used procedure is that described by Schneider in 1948 (50). Recently with the development of the Spinco type of refrigerated ultracentrifuge much higher speeds are obtained (51,52) and a better **separation** of submicroscopic particles has been accomplished.

Before starting the fractionation experiments, it was thought pertinent to investigate the distribution of alkaline phosphatase throughout the entire small intestine. This would give a more complete picture of the localization of the enzyme and would indicate the selection of the most active section for the fractionation studies.

Finally the kinetics of the reaction of the alkaline phosphatase of the various fractions with β-glycerophosphate were investigated.

EXPERIMENTAL

Adult male albino rats were used in all experiments. They were maintained on Purina fox checkers and water ad libitum. They were killed by decapitation as described in the general introduction. For the distribution studies, the entire small

intestine from the pylorus to the ileocolic valve was taken
out, cut in sections 10 cm. long, which were then placed in
individual small beakers kept in ice water. The various
sections were cleared from adjacent tissues and washed with
isotonic sucrose solution. The lower parts were especially
difficult to clean. The contents of these sections were
adherent and it was impossible to wash them away. A glass
rod was usually employed to remove the material from the lumen.
A good rinsing with isotonic sucrose solution followed. The
sections were subsequently dried, weighed and homogenized as
described on pages 6 and 7. The homogenates were diluted
with 0.25M sucrose as follows:

      First 5 sections; 1:6,250 (1:25:250)

      6th-9th section inclusive; 1:2,500 (1:25:100)

      10th-12th section inclusive; 1:625 (1:25:25)

For the fractionation and kinetic studies only the first
10 cm. from the pylorus were used. This portion was prepared
as described on page 6. The homogenization was carried out
with isotonic sucrose and the volume made up to 25 ml.

Fractionation was performed according to the method of
Schneider (50), slightly modified in order to obtain a nuclear
fraction less contaminated by mitochondria. An International
refrigerated centrifuge (Model PR-1) was used for this purpose.
All operations were carried out at 2°-3°C. Glassware and
lusteroid tubes were always prechilled.

The gravitational force obtained each time was calculated
by the formula:

$$F = \frac{W^2 r}{980} = \frac{S^2 r}{89,500}$$

where F = centrifugal force (in gravitational units)

W = angular velocity

r = radial distance in cm. from the centre of

rotation to the centre of the solution.

S = speed in revolutions per minute.

Twenty ml. of the homogenate were pipetted into two 15 ml. lusteroid tubes and were centrifuged at 600xg (2,000 r.p.m, centrifuge head no. 824) for 5 minutes. Nuclei, unbroken cells, red blood cells, and a few of the heavier mitochondria were sedimented to the bottom of the tube. The supernatant fluids were poured into two 35 ml. lusteroid tubes and the sediments were rehomogenized with 3 ml. of sucrose. They were recentrifuged at the same speed and for the same length of time and this was repeated once more. The final sediment contained the nuclei but a few mitochondria were still present.

The combined supernatant fluids were centrifuged at 8,500xg (12,300 r.p.m.: Multispeed attachment No. 2551, head 296) for 10 minutes. The new supernatants were again collected in 35 ml. lusteroid tubes and the sediments were washed twice with 3 ml. of isotonic sucrose. This fraction represented the mitochondria and on microscopic examination it was found to be homogeneous.

The supernatant fluids, collected after the removal of the mitochondria, were centrifuged for 90 minutes at 16,500xg (12,300 r.p.m.), which was the greatest gravitational force one could attain without breaking the tubes. The last 4 experiments were carried out in a Spinco refrigerated centrifuge

(Model E preparatory Rotor K) and 63,434xg and 113,380xg were employed for 60 and 40 minutes respectively (speeds = 27,960 and 37,000 r.p.m.). The sediments were washed once with isotonic sucrose solution. These represented the "microsomal" fraction. In dark field microscopy they seemed to be composed of bright pin point particles with rapid Brownian movement. They appeared homogeneous.

The various sediments were suspended in isotonic sucrose solution. They were further diluted according to their phosphatase activity to the following final dilutions:

Nuclear and Mitochondrial fractions:	1:780
Microsomes	1:6,250
Supernatant fluid	1:3,125
Unfractionated homogenate	1:12,500

Total nitrogen determinations were carried out by the titrimetric semi-micro Kjeldahl method of Hiller, Plazin and Van Slyke (53).

RESULTS AND DISCUSSION

(a) DISTRIBUTION

The length of the small intestine varies from rat to rat. Measurements obtained from 20 animals gave a mean length of 116 ± 1 cm. In the present experiment with 5 rats, however the lengths happened to range from 100-125 cm. Table I and Figure 1 represent the results.

Table I

Phosphatase Activity Exhibited by the Various 10 cm. Sections

of the Small Intestine of Adult Male Albino Rats (Wistar Strain)

Results are averages of 5 animals

Section Number	Phosphatase activity, units per 100 gm. wet intestine	Percentage of sum of the 12 sections	Log. of percentage activity
1	22,800 $\pm$ 1,760 *	21.8 $\pm$ 1.7 *	1.338
2	17,800 $\pm$ 1,880	17.0 $\pm$ 1.8	1.230
3	14,570 $\pm$ 900	13.9 $\pm$ 0.8	1.143
4	11,700 $\pm$ 784	11.2 $\pm$ 0.8	1.049
5	10,200 $\pm$ 1,640	9.7 $\pm$ 1.6	0.987
6	8,900 $\pm$ 1,100	8.5 $\pm$ 1.0	0.989
7	6,100 $\pm$ 1,300	5.8 $\pm$ 1.3	0.763
8	4,000 $\pm$ 1,200	3.8 $\pm$ 1.2	0.578
9	3,300 $\pm$ 1,220	3.2 $\pm$ 1.2	0.505
10	2,000 $\pm$ 310	1.9 $\pm$ 0.3	0.279
11	1,860 $\pm$ 80	1.8 $\pm$ 0.1	0.255
12	1,580 $\pm$ 160	1.5 $\pm$ 0.2	0.176
Total	104,800 $\pm$ 4,100	100.0 $\pm$ 3.9	

* Standard error of the mean.
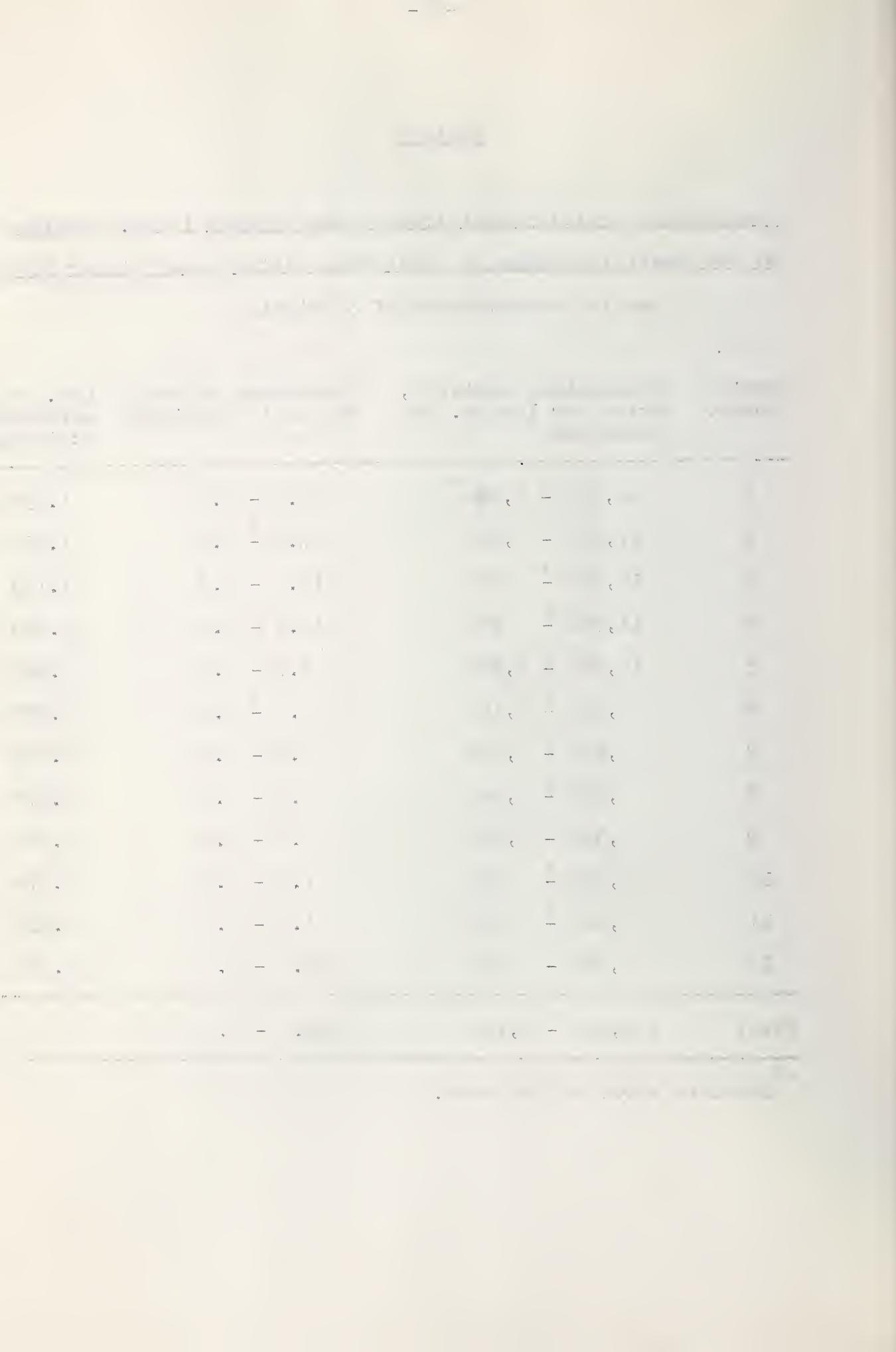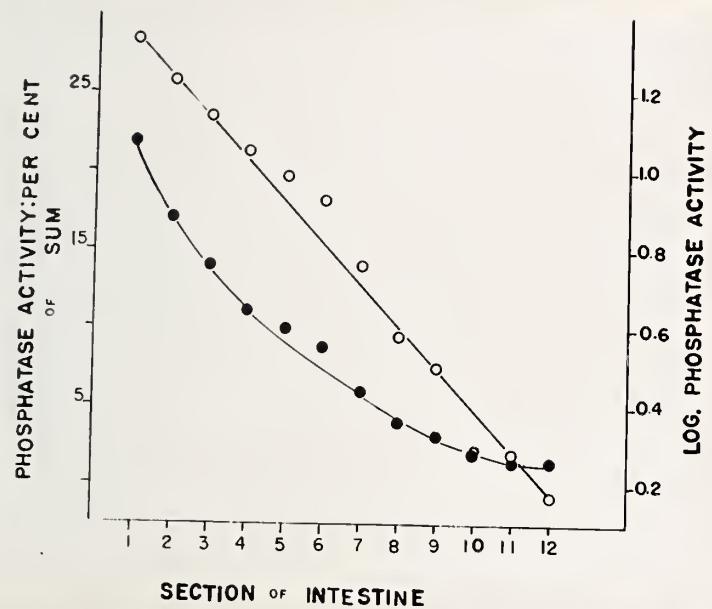
Figure 1. Distribution of the alkaline
phosphatase in the small intestine of the rat
The sum of the activities of all sections
loo per cent. (●——●)=per cent phosphatase
activity. (o—o)=log per cent phosphatase
activity.

It is apparent that the levels of the alkaline phosphatase
are reduced exponentially from the duodenum to the end of the
ileum. The rate of decrease, calculated from the regression
line in Figure 1, is 0.1105[*] of the log of phosphatase activity
per section.

The deviations observed in the figure are mainly due to
differences in length among the 5 intestines. It is obvious
that the classification of the intestine per 10 cm. sections
did not put together strictly homologous fractions from each
intestine; the discrepancy increases, the farther we go from
the pylorus. A better way of investigation would probably be
to examine activity per fraction of intestine: e.g. divide
each intestine in tenths, independently of the actual length
of the fractions of each intestine. This would permit a
better classification. The slope of the line and consequently
the rate of decrease would be slightly different then.

Another source of error is the elasticity of the tissue.
The length of the gut changes easily depending on the degree
of contraction of the muscular coat and the tension which one
applies when he is working with it. This can affect the
classification of the various parts of the small intestine
obtained from any one rat.

The results in Table I are expressed on the basis of
weight. However, there is a progressive decrease in the
weight of the small intestine from the pylorus down. If the
phosphatase values in Table I are changed to activity of the
enzyme/section, the rate of decrease is even more marked.

[*]
The regression equation computed from the experimental data is,
$\hat{y} = 0.1105x + 0.051$

Thus the value for the duodenum (section 1) increases to 26%
of the total alkaline phosphatase activity/small intestine,
while the value for the last part of the ileum (section 12)
goes down to 0.7% (total activity = 606 $\pm$ 24 units of alkaline
phosphatase/small intestine). It is well established that
digestion and absorption are more pronounced in the beginning
of the small intestine and they slow down gradually in the
terminal regions. Is it coincidental that their rate decreases
~~nearly~~ as phosphatase activity decreases; are the increased
amounts of phosphatase in the initial parts of the intestine
one of the reasons for the higher absorptive ability of the
intestine in these sections or is the contrary true?
Cytochemical studies have shown that the striated border of
the columnar epithelium is rich in phosphatase (54,55,56,57).
Any biological material taken per os has to pass through this
striated border in order to be absorbed so the possibility is
high that it will come in contact with phosphatase during this
process.

(b) FRACTIONATION STUDIES

It is evident from Tables II and III that: (a) all
fractions have a certain degree of phosphatase activity.
(b) the "microsomes" have the highest activity of all: 76%
of the total, i.e. about 7.2 times more than the nuclei, 10
times more than the mitochondria and 8 times more than the
supernatant fluid. Their specific activity is 51.6 i.e. 6.15
times higher than that of the total homogenate. This clearly
indicates that alkaline phosphatase is definitely associated
with these cellular particulates. (c) Good recoveries are
obtained, with no indication of inhibition or activation (43).

Table II

Fractionation of Intestinal Homogenates

("Microsomes" centrifuged at 16,500 x g for 90 minutes)

Results are averages of 6 rats

Fraction	Units of Phosphatase activity per 100 gm. of wet intestine	Percentage of the total homogenate activity	Nitrogen, mgm. per 100 gm. of wet intestine	Specific activity= Phosphatase units mgm. of N
Nuclei	2,190 ± 140 *	10.5 ± 1.1 *	760 ± 41 *	2.9 ± 0.2 *
Mitochondria	1,760 ± 220	7.5 ± 1.0	160 ± 12	9.6 ± 0.9
Microsomes	13,150 ± 420	64.1 ± 1.9	260 ± 25	52 ± 4
Supernatant	3,530 ± 270	17.2 ± 1.3	1,350 ± 39	2.6 ± 0.6
Unfractionated homogenate	21,400 ±1000	Recovery 99.3 ± 4.7 unfrac homog.	2,520 –130	8.4 ± 0.6

* Standard error of the mean
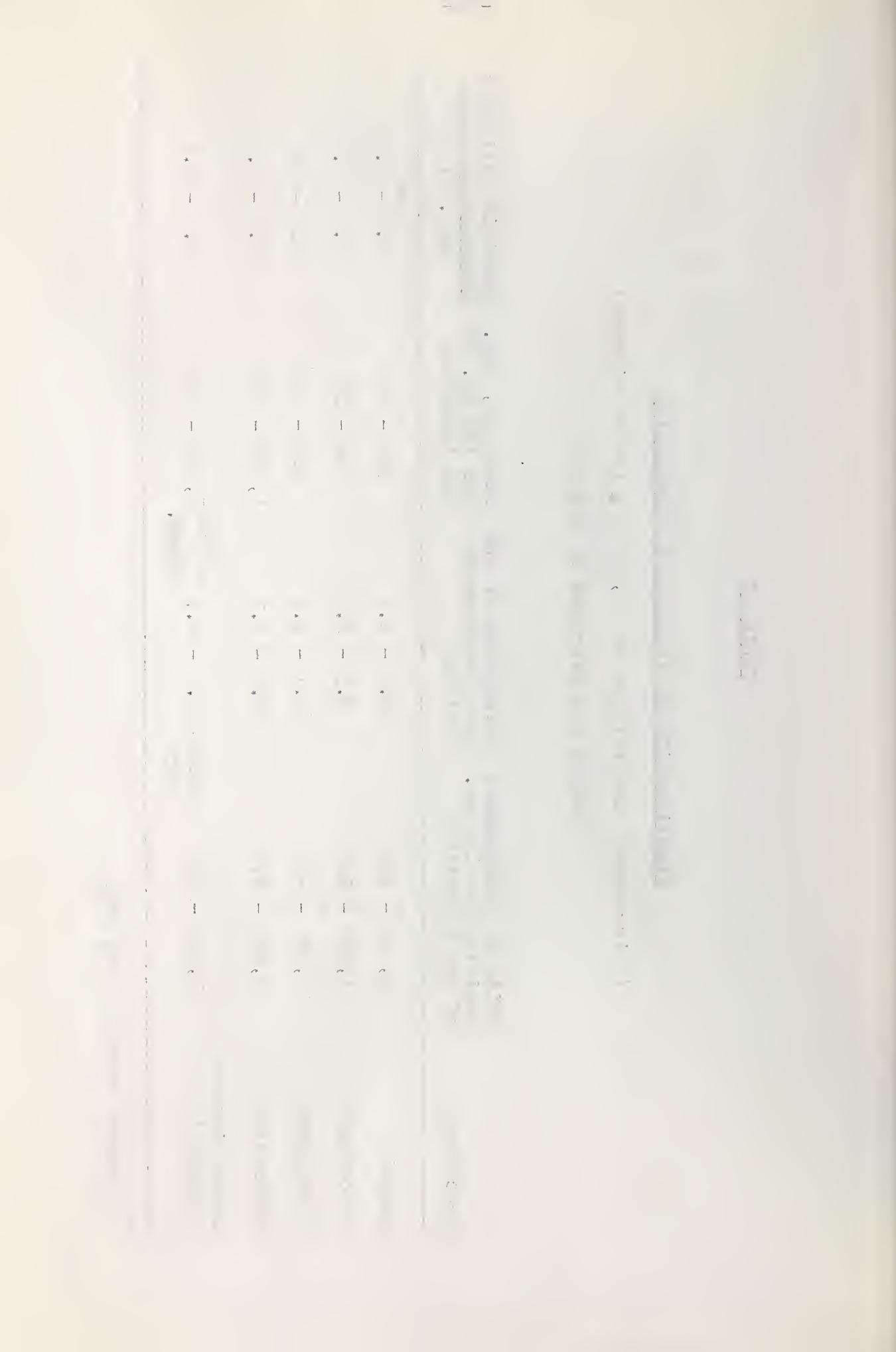
Table III

Sedimentation of "Microsomes" from Intestinal Homogenates

(At 63,434 x g for 60 minutes, washed and recentrifuged
at 113,380 x g for 40 minutes)

Fraction	Units of Phosphatase per 100 gm. of wet intestine	Percentage of the total homogenate activity
Nuclei + Mitochondria	2,800	15.1
Microsomes	$14,000 \pm 1,800$ *	76 ± 10 *
Supernatant	$1,840 \pm 60$	9.4 ± 0.4
Unfractionated homogenate	$18,500 \pm 500$ Recov.	100.2 ± 2.7

* Standard error of the mean

The actual activity of the microsomal fraction within the cell may be greater than 76%. It was noted in previous experiments that if the amount of isotonic sucrose used to wash the various fractions was increased to more than 3 ml., the phosphatase activities of the respective fractions was diminished, while that of the supernatant was increased accordingly. Thus the activities of the nuclear and the mitochondrial fractions were observed to be reduced to 2.4 and 3.9% respectively. It seems that some phosphatase is washed out of the microsomes by the **sucrose**. In the experiments performed with the Spinco refrigerated ultracentrifuge, the tubes had to be filled completely with solution every time in order to be able to withstand the high gravitational forces developed during centrifugation. As a consequence, the microsomes were suspended in large amounts of sucrose each time (35 ml.). Some phosphatase molecules may have diffused into the medium.

de Duve and associates used small amounts of washing fluid and in some instances they did not wash the "microsomes" at all. Although they applied lower gravitational forces (20,000xg for 90 minutes) they found that 97% of the phosphatase activity of the intestine of the rabbit and 83% of the activity of the intestine of the guinea pig was associated with the "microsomes" (58). In the latter case, the microsomes were unwashed. This clearly shows that besides the washing factor there is a species difference as well.

Recently with the development of electron microscopy, it has been shown that the "microsomes" are not independent entities, as it was thought they were, but the fragments of the endoplasmic reticulum, which has been extensively broken down during homogenization (59). As the main components of such a complicated and widely distributed system within the cell, they must be very important to cellular economy. They have long been implicated in protein synthesis and the question arises; has phosphatase, which is so abundantly associated with them, anything to do with this process? Protein synthesis is one of the main functions attributed to the "microsomes".

(c) KINETICS

(1) Determination of the reaction order.

Results are represented by Figure 2. It is obvious that the reaction is one of a zero order for the first 60 minutes for the unfractionated homogenate as well as for the various fractions. The reaction constants (K_o) for the concentrations used are:

Nuclei: 11.8×10^{-2} γ Phosphorus/minute

Mitochondria: 5.8×10^{-2} γ Phosphorus/minute

Microsomes: 6.6×10^{-2} γ Phosphorus/minute

Supernatant: 8.3×10^{-2} γ Phosphorus/minute

Unfractionated homogenate: 19.4×10^{-2} γ Phosphorus/minute

(2) Optimum pH.

The results are plotted in Figure 3. The effect of the pH of the incubation mixture on the activity of the intestinal
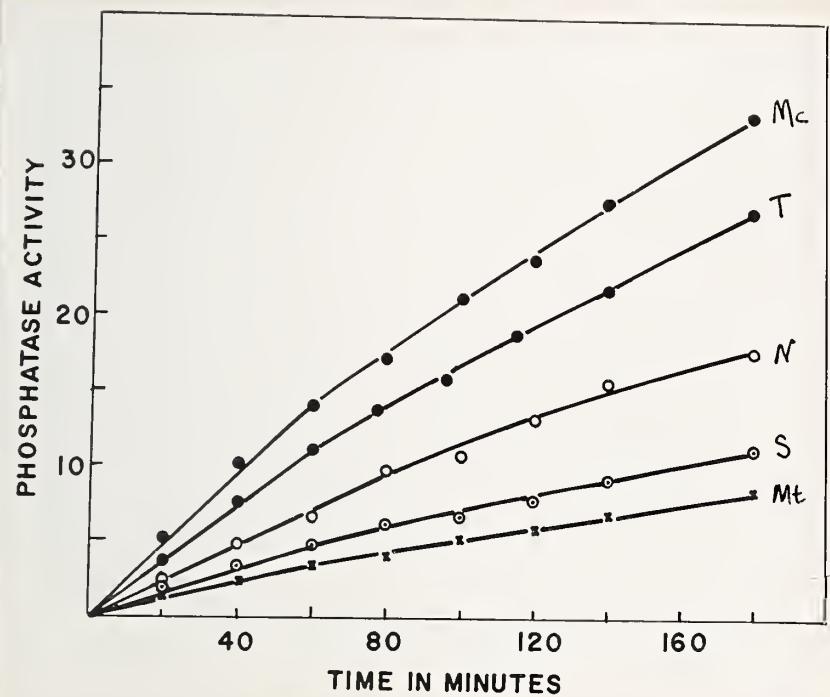
Figure 2. Effect of time of incubation on
intestinal alkaline phosphatase activity
(mg P per incubation mixture). Mc = micro
somes. T = total homogenate. N = nuclei. S =
=supernatant fluid. Mt = mitochondria.

alkaline phosphatase of the rat is the same for all cellular

fractions with the optimum point at pH 9.3. This subject

will be discussed further in the last division of this thesis.

Small amounts of acid phosphatase are also present in

the cellular fractions from the intestine. Determinations

at a pH 4.9 with lower dilutions of homogenates (31.25-625

times) gave the results represented by Table IV.
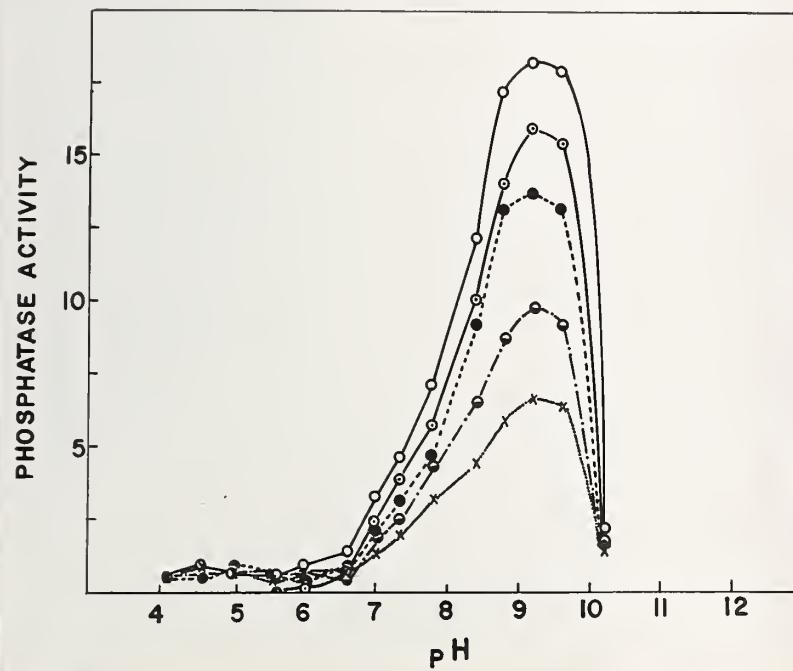
Figure 3. Effect of pH of the hydrolysis mixture
on the activity of the intestinal alkaline phosphatase
of the rat (mgP per hour per hydrolysis mixture)(o—o)
= total homogenate (⊙—⊙)= microsomes, (●---●)= super-
natant fluid, (◐--◐)= mitochondria, (x····x)= nuclei.

Table IV

Distribution of the Acid Phosphatase Activity of the Various

Cellular Fractions of the Intestine of the Albino Rat

(Results are averages of 5 animals)

Fraction	Acid Phosphatase activity units/100 gm. of wet intestine	Percentage of the total homogenate activity	$\frac{Acid}{Alkaline}$ x 100
Nuclei	24 ± 2 *	11 ± 1	1.1 ± 0.1
Mitochondria	39 ± 6	18 ± 3	2.5 ± 0.6
Microsomes	86 ± 9	40 ± 1	0.63 ± 0.1
Supernatant	70 ± 1	33 ± 1	1.2 ± 0.2
Unfractionated homogenate	212 ± 6.0 Recovery	102 ± 3 Total homog.	0.96 ± 0.1

* Standard error of the mean
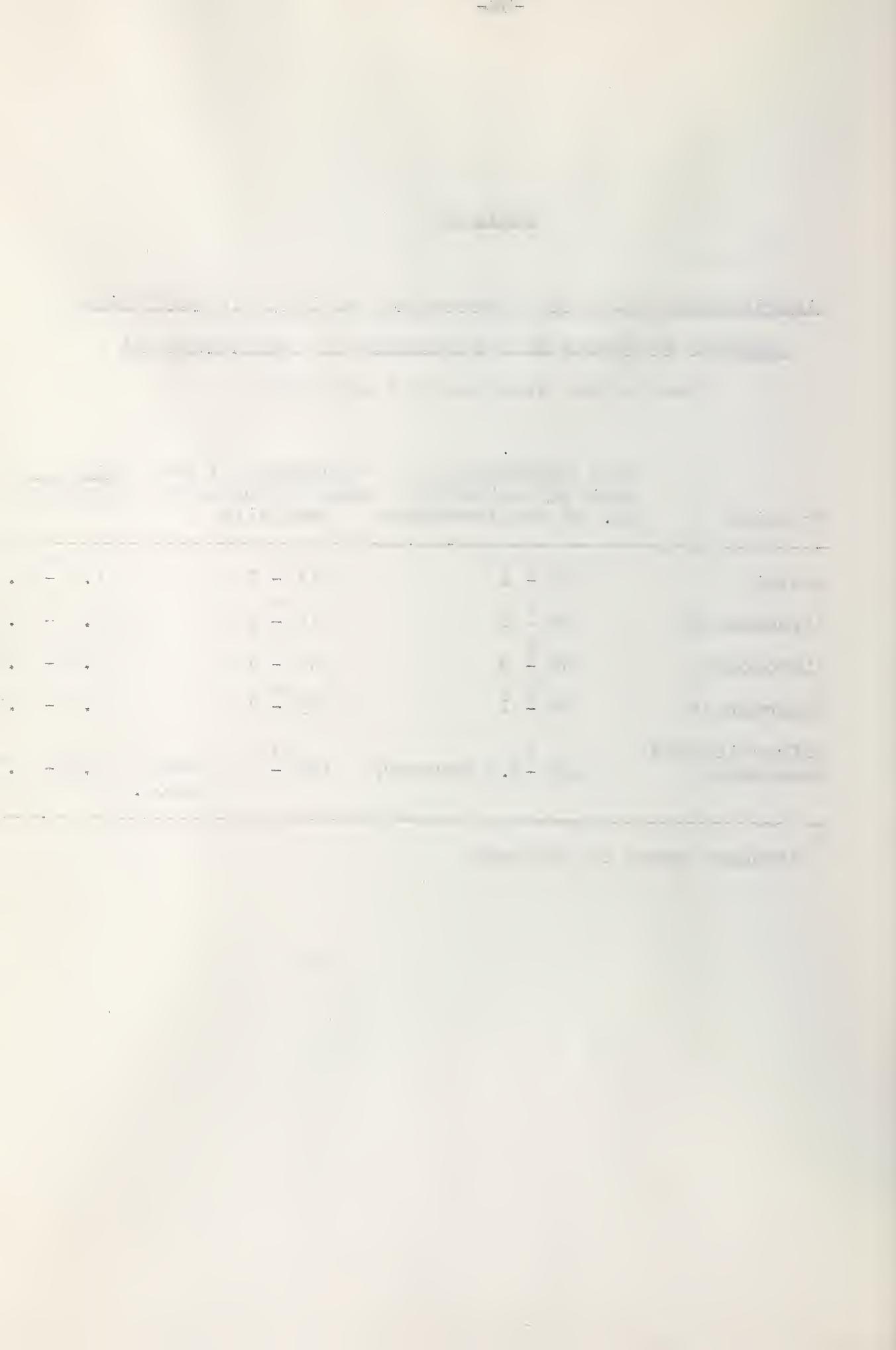
The microsomal fraction again seems to exhibit the highest percentage of phosphatase activity. Berthet and de Duve (60) worked with liver homogenates and found that 56% of the acid phosphatase is associated with the mitochondria but in fresh liver homogenates it is mainly bound and does not act on glycerophosphate. The enzyme is released after aging or after repeated freezing and thawing.

(3) Michaelis constant (Km)

The effect of substrate concentration on the reaction rate was studied next. Solutions of β-glycerophosphate were prepared which gave concentrations in the range from 0.0013M to 0.0266M in the hydrolysis mixture. The concentration of Mg^{++} and sodium diethylbarbiturate were the same as in the original Shinowara method (i.e. Mg = 0.0067M; barbiturate = 0.0106M/hydrolysis mixture). The results are plotted in Figures 4a,4b.The Michaelis constants were determined from the experimental data by means of the following equations:

$$\sum \frac{1}{v} = \frac{n}{Vmax} + \sum \frac{Km}{Vmax} \frac{1}{(s)}$$

$$\sum \frac{(s)}{v} = \frac{nKm}{Vmax} + \sum (s) \cdot \frac{1}{Vmax}$$

where v = velocity

n = number of substrate concentrations used

Vmax = maximum velocity

(s) = substrate concentration

The values obtained for the Km of the different fractions are:

Unfractionated homogenate: 0.0041M

Nuclei: 0.0034M

Mitochondria: 0.0030M

"Microsomes":   0.0025M

Supernatant fluid: 0.0023M

The slight differences in the values of Km cannot

justify the assumption of the presence of a different

phosphatase in each fraction. If this were the case,

the Km of the unfractionated homogenate ought to be the

weighted mean of the Kms of all fractions, a value very

close to the Km of the microsomal fraction once this

fraction has been found to exhibit 76% of the total

phosphatase activity. The good recoveries obtained in

section (b) of this chapter exclude the possibility of any

mutual inhibition or activation by other factors of

supposedly different enzymes in the various fractions.

It was noticed that the degree of homogenization had

some influence on the value of Km of the total homogenate.

Michaelis constants which were determined on crude

homogenates, were found to be slightly higher than the

Km values obtained for the same homogenates after they had

been subjected to further treatment in the glass homogenizer.

It seems plausible that these slight differences in the

values of Km of the various fractions may be due to a difference

in the degree of availability of the substrate for the enzyme

in the various fractions. The reaction between enzyme and

substrate is usually represented by the following equation:

$$E + S \xrightarrow[K_2]{K_1} ES \xrightarrow{K_3} P + E$$
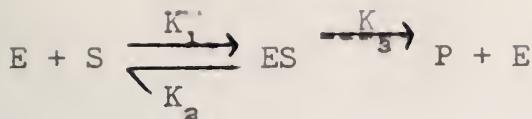
where E = enzyme

ES = enzyme-substrate complex

P = products

The Michaelis constant actually determined from the experimental data represents: $Km = \dfrac{K_2 + K_3}{K_1}$

The constant, K_1, is dependent on: (a) the affinity of the enzyme for the substrate (b) the frequency of collisions between them. In the case of the nuclei and mitochondria, the substrate has to get through the nuclear and mitochondrial membranes first in order to react with the enzyme; this will have a retarding effect on K_1 and Km will become larger.

Although the "microsomes" are regarded no longer as entities (59) they do possess a certain structure. They are fragments of membranes with small particles attached to them. The fact that 76% of the phosphatase activity in rat intestine is associated with the "microsomes" is presumptive evidence that the enzyme is bound to them. The substrate now does not have to cross a complete membrane. Therefore the effect on Km is much slighter.

The phosphatase in the supernatant fluid is mostly in the free form, and consequently the Km of this fraction has the lowest value.

In crude homogenates, many of the cells are intact. This means that the substrate has to cross the cellular membrane as well, in order to be available for the reaction. This results in further delay and further increase of Km.
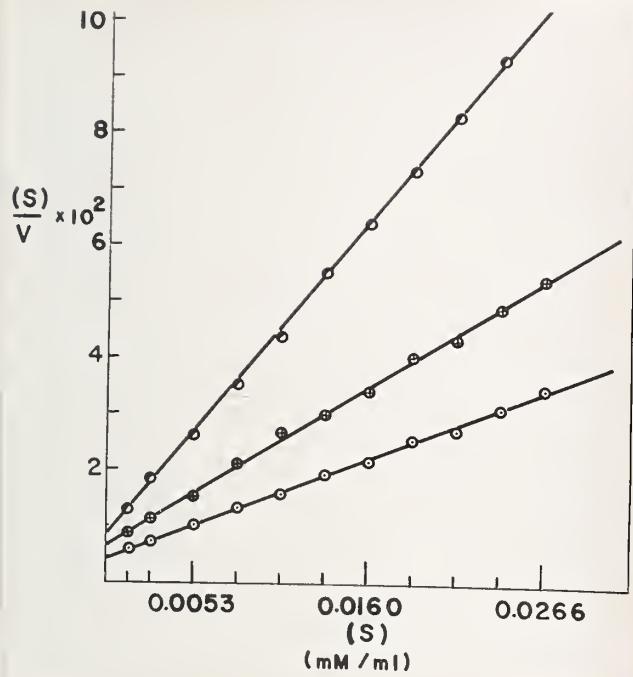
Figure 4a. Effect of substrate concentration on the activity of the intestinal alkaline phosphatase of the rat. (●)=microsomes, (⊗)=nuclei , (⊙)=total homogenate.
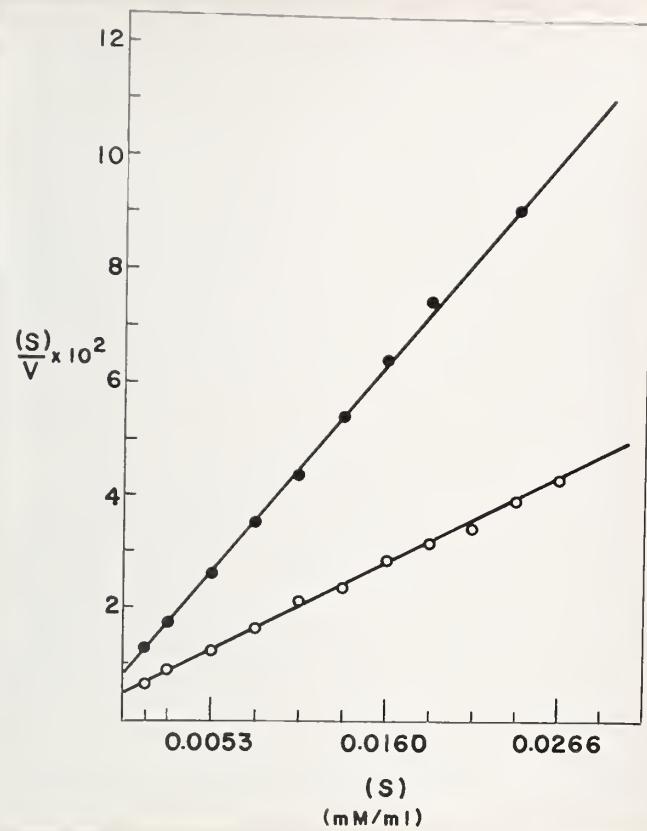
Figure 4b. Effect of substrate concentration
on the activity of the intestinal alkaline
phosphatase of the rat. (●—)=supernatant
fluid , (o—)=mitochondria.

(4) Energy of Activation

The degree of the hydrolysis of sodium β-glycerophosphate by the phosphatase of the various fractions was studied at the following temperatures (C°) for the time periods indicated:

2.7° for 60 minutes

16.2° for 40 minutes

20.2° for 30 minutes

24.2° for 30 minutes

28.8° for 25 minutes

33.0° for 20 minutes

37.0° for 15 minutes

41.0° for 10 minutes

45.0° for 5 minutes

Shorter periods of time were used at the higher temperatures in order to avoid inactivation of the enzyme. Longer incubation periods at the lower temperatures were justified by the desire to secure an accurately measurable amount, of hydrolysis products. The rate of the reaction does not change with time, (even after considerable periods) when Mg^{++} is present in the incubation mixture (61,62) and as long as there is no enzyme inactivation and the hydrolysis does not exceed 10% of the substrate.

A preliminary experiment was done in order to study the possibility of the inactivation of the enzyme at the selected temperatures under the conditions of the experiment. For this reason 0.5 ml. of a buffer solution containing 0.0274M sodium-diethyl barbiturate and 0.0134M $MgSO_4$ was added to

0.5 ml. of homogenate (both solutions had been previously brought to the required temperature), and incubated for the desired length of time at pH 9.2-9.4. The tubes were then placed quickly in ice-water after incubation to prevent further enzyme inactivation, if any. Finally, the phosphatase activity of the contents of each tube was determined at 2.7° for 60 minutes by adding 0.5 ml. of substrate solution which contained 0.0318M sodium-β-glycerophosphate, 0.0137M barbiturate and 0.0067M MgSO$_4$ (final concentrations: sodium-β-glycero-phosphate, 0.0106M; barbiturate, 0.0137M; MgSO$_4$: 0.0067M per hydrolysis mixture, as in the original method). The results are tabulated in Table V.

From Table V it seems that the enzyme activity was unimpaired at any temperature, during the specified length of time for each, in the above experiment.

For the determination of the energy of activation 2 sets of experiments were performed. In the first, the following temperatures (C°) were used:

2.7° for 60 minutes

16.2° for 40 minutes

20.2° for 30 minutes

28° for 20 minutes

37° for 15 minutes

In the second set of experiments, which were done two weeks later with newly prepared solutions, different pipettes, and newly prepared cellular fractions, the hydrolysis was

Table V

Test of the Stability of the Intestinal Alkaline
Phosphatase of the Albino Rat at Various Temperatures

Temperature	γP per ml. hydrolysis mixture per hour	Deviation from mean
2.7°	3.08	+ 0.006
18.2°	3.12	+ 0.046
20.2°	2.98	− 0.094
24.2°	3.08	+ 0.006
28.8°	3.13	+ 0.056
33.0°	2.98	− 0.094
37.0°	3.18	+ 0.106
41.0°	3.13	+ 0.056
45.0°	3.02	− 0.054

Note average percent error above was 1.8.

carried out at the temperatures listed below:

24.2°C for 30 minutes

28.8°C for 25 minutes

33.0°C for 20 minutes

37.0°C for 15 minutes

41.0°C for 10 minutes

45.0°C for 5 minutes

The energies of activation for all the fractions were calculated both from the slope of the line after plotting log K vs. 1/T and by using the integrated form of the Arrhenius equation:

$$\ln K = \frac{EC}{R} - \frac{E}{R} \frac{(1)}{(T)}$$

where: K = reaction constant at the absolute temp., T.

R = gas constant in calories (1.987 cal.)

E = energy of activation

T = absolute temperature

C = integration constant

Table VI contains the mean values of energies of activation derived from both sets of experiments and Figure 4 is the graphical representation of energies of activation obtained by the second set of experiments.

In general, the differences between the results obtained from the slope of the line (log K vs. 1/T) and those obtained from the Arrhenius equation are within the range of experimental errors. (Average error = 1.6%)

Table VI

Energies of Activation of the Intestinal Alkaline Phosphatase of the Various

Fractions

Fractions	E, from Arrhenius Equation	E, from Slope
Nuclei	10,930 ± 110*cal/gm. mole	11,200 ± 300*cal/gm. mole
Mitochondria	11,720 ± 260 cal/gm. mole	11,600 ± 300 cal/gm. mole
Microsomes	11,800 ± 160 cal/gm. mole	11,800 ± 200 cal/gm. mole
Supernatant	11,300 ± 130 cal/gm. mole	11,550 ± 50 cal/gm. mole
Total Homogenate	11,300 ± 280 cal/gm. mole	11,250 ± 250 cal/gm. mole
Average	11,416 ± 160 cal/gm. mole	11,470 ± 130 cal/gm. mole

* Standard error of the mean
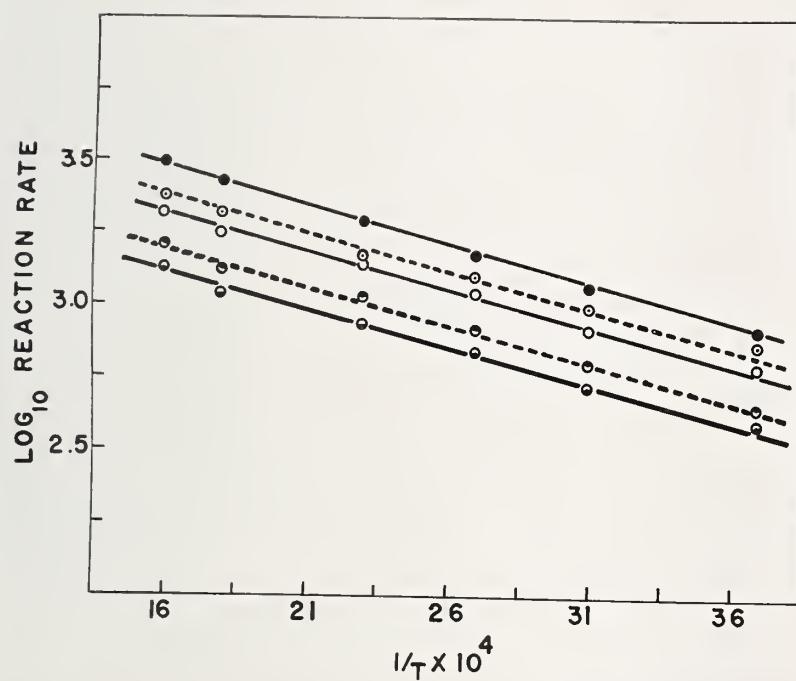
Figure 5. Effect of temperature on the reaction
rate of the intestinal alkaline phosphatase acti-
vity. (●—●) = microsomes , (☉--☉) = nuclei ,
(○—○) total homogenate , (●---●) mitochondria,
(◐—◐) supernatant fluid.

Bodansky (63) found that the energy of activation for bone phosphatase is $9,940 \pm 140$ cal/gm. mole. This does not necessarily mean that the two enzymes are different. The substrate used by Bodansky contained 0.00625M glycine per hydrolysis mixture besides Mg^{++}. Glycine in this concentration (61,62) has a considerable activating effect on alkaline phosphatase. It is not surprising therefore, that a lower activation energy is required in this case.

Later, well after these experiments were over, it was discovered that the chlorine present in the double distilled water (64) had an inhibiting effect on intestinal alkaline phosphatase, although it did not affect the blood enzyme. This, of course, is an additional reason for higher activation energy.

Bodansky (62) has also reported that the amino acids liberated during the autolysis of the intestine, have a retarding effect on alkaline phosphatase. This cannot be the case here. The homogenates used in all these experiments were always fresh, not older than 48 hours, and kept continuously in the cold room. Autolysis was not so pronounced as in the case of Bodansky's preparations, which were left at room temperature for several days. The original amino acid concentration of our homogenates was 2.60 ± 0.06 x 10^{-6}M per hydrolysis mixture. Even if we assume that there was a two-fold increase in two days, the levels still would remain well below the values given by Bodansky as inhibitory $(1 \times 10^{-3} M)$.

Madsen and Tuba (65) attempted to determine the energy
of activation of alkaline phosphatase in homogenates of the
rat intestinal mucosa. Their value of F was 13,900 cal/gm.,
mole for the hydrolysis of sodium β-glycerophosphate.
However, only 4 temperatures were used in the above
investigation, and at one of these, 37°C, inactivation of the
enzyme appeared, and data for this temperature had to be
excluded from calculations for the value of E.

(5) Mg^{++} Activation

The effect of Mg^{++} concentration on the alkaline
phosphatase activity of the various fractions of rat
intestinal homogenate is shown in Table VII and Figures
6a and 6b. It is quite clear that Mg^{++} has a strong
activating effect on all fractions. The rate of increase
in activity is quite high at low Mg^{++} concentrations. The
maximum activation is reached relatively quickly and remains
constant over a wide range of concentrations (6.7×10^{-4} -
2.0×10^{-2} M) after which the degree of activation becomes
continuously smaller with increasing Mg^{++} concentration.
It is evident from the table that the percent increase in
rate varies for the different fractions and unfractionated
homogenate. The nuclear alkaline phosphatase shows the
least activation, while the supernatant fluid enzyme exhibits
the greatest response to Mg^{++}. Nevertheless, the pattern is
much the same in all of them.

The activating effect of Mg^{++} was first discovered by

Table VIl

Effect of Magnesium Ion Concentration on the Alkaline Phosphatase Activity of the Various Fractions of Rat Intestinal Homogenate

Magnesium concentration

Cellular Fraction	6.7×10^{-5}	3.3×10^{-4}	6.7×10^{-4}	3.3×10^{-3}	6.7×10^{-3}	1.33×10^{-2}	2.0×10^{-2}	3.3×10^{-2}	6.7×10^{-2}
Nuclei	164*	176	182	181	180	182	188	155	145
Unfractionated Homogenate	175	191	204	193	207	204	210	175	168
"Microsomes"	182	199	209	195	210	202	226	188	170
Mitochondria	192	246	231	221	231	234	242	195	178
Supernatant Fluid	219	250	259	234	259	254	270	225	209

* Values are percentages of the reaction rate obtained in the absence of Magnesium.
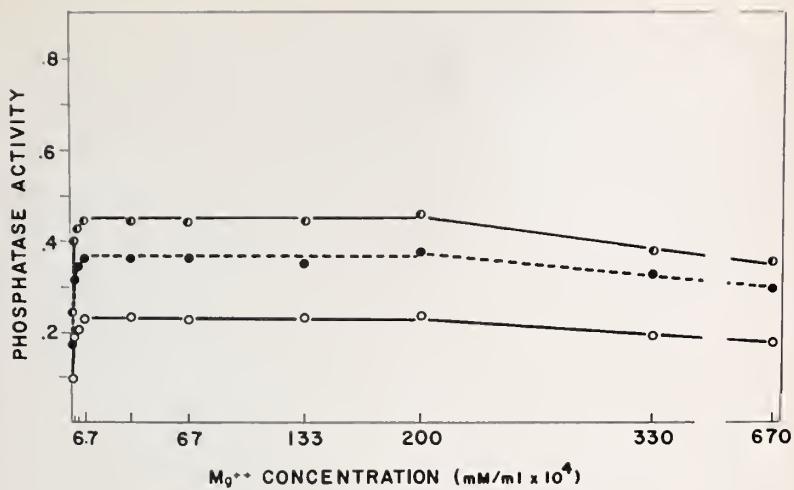
Figure 6a.(●—)=nuclei, (●---)=microsomes,

(o—)=mitochondria.
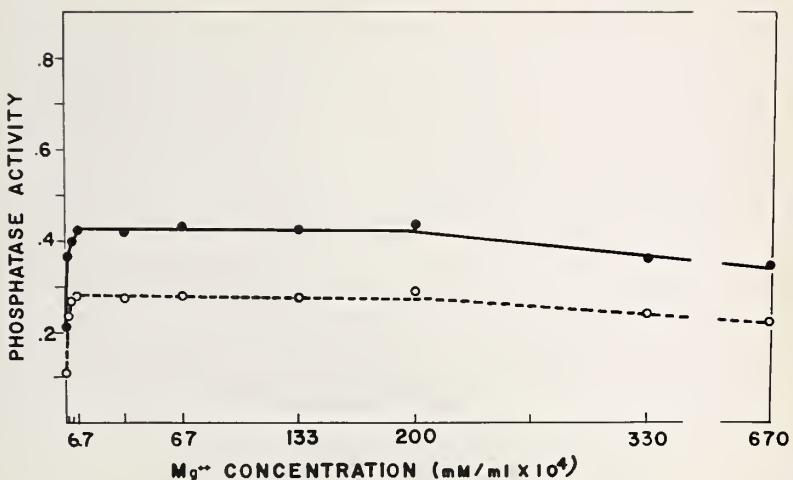
Effect of Mg++ concentration on the activity

of Intestinal Alkaline Phosphatase (mg P per

hour per hydrolysis mixture.)

Figure 6b. (●—)=total homogenate, (o---)=super-

natant fluid.

Erdtman (66) in 1927 and was subsequently studied by
Hommerburg (67), Jenner and Kay (68), Bakwin and Bodansky (69).
Jenner and Kay introduced the term qMg which is the negative
logarithm of the molar concentration of Mg^{++}. These authors
obtained a bell shaped curve when they plotted reaction
rate vs. qMg. The plateau corresponded to 2-3 qMg i.e. 1 x
10^{-2} - 1 x 10^{-3} M, which is about the same as that observed
by us. The activation is due to the formation of a
coordination complex between Mg^{++}, enzyme and PO_4 group of
substrate; thus the activated state of the enzyme is
stabilized (70). In the presence of high Mg^{++} concentration,
the conditions may favor the formation of a different type
of complex, probably with 2 molecules of Mg^{++}, which is less
active or not active at all, and thus the amount of activated
enzyme is reduced. A slight fall in the pH of the reaction
mixture after incubation was noted in the presence of high
Mg^{++} concentrations. This may be another (or the only)
reason for the decrease in activation rate observed at these
concentrations.

The difference in sensitivity of the enzyme of the
various fractions towards Mg^{++} may be due to the different
way and degree of attachment of the enzyme to these fractions,
so that the availability of the enzyme to Mg^{++} is different in
each case. In the supernatant fluid, the enzyme is more or
less free, thus the Mg^{++}-enzyme protein-substrate complex is
formed easily and maximum activation occurs. Finally, the
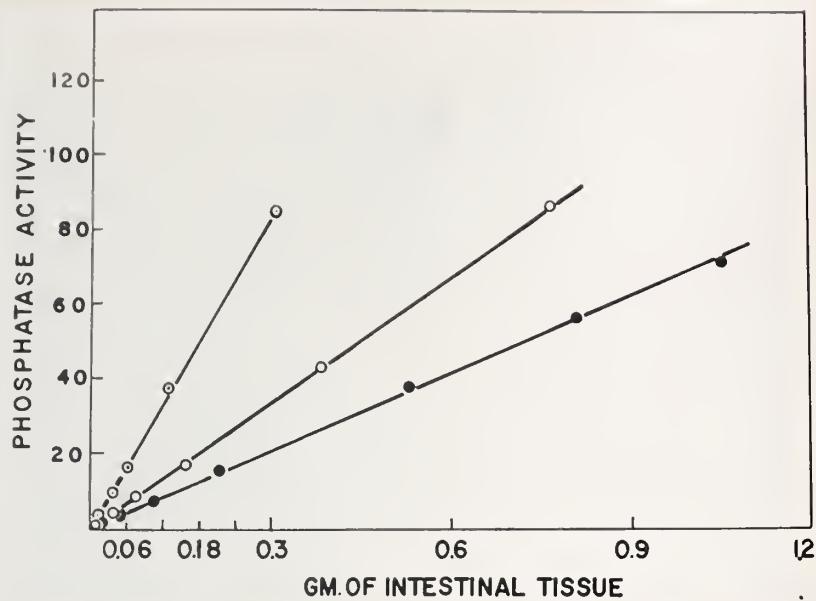
Figure 7a.(⊙)=total homogenate, (o)=microsomes,
(•)=supernatant.
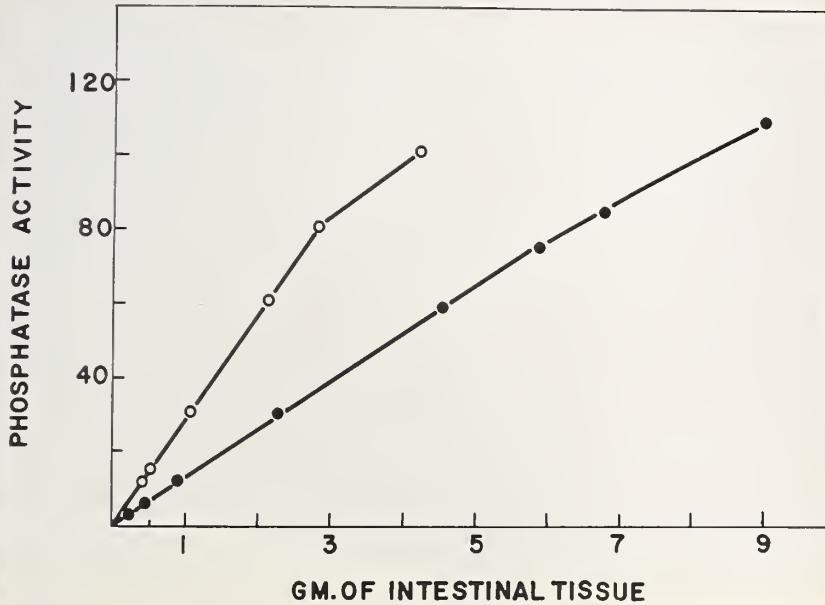
Effect of the enzyme concentration on the rate
of the reaction of Intestinal Alkaline Phospha-
tase of the rat.(mg P/hour/hydrolysis mixture).
Figure 7b. (o)=nuclei, (•)=mitochondria.

presence of other substances in the various fractions
competing for Mg^{++} may be another factor for the observed
difference in sensitivity.

(6) Enzyme Concentration

The effect of the enzyme concentration on the reaction
rate is represented by Figure 7a, 7b. The behavior of all
fractions as far as enzyme concentration is concerned, is
identical with that of the unfractionated homogenate. As
long as the concentration of the enzyme does not exceed
80 units per litre of homogenate, a straight line is
obtained by plotting activity vs. enzyme concentration
(gm. of intestinal tissue). In higher concentrations, the
rate is lower than that expected from direct proportionality.
The reasons may be: (a) The amount of substrate present
with high enzyme concentrations is not adequate to saturate
all the enzyme molecules (b) hydrolysis exceeds 10% of the
substrate and the accumulation of the hydrolytic products
favors the opposite reaction (c) the possibility of the
presence of inhibiting factors in the intestine cannot be
excluded. They may not be active at low concentrations, but
may retard enzyme activity when such inhibitors are present
in large amounts.

SUMMARY

It may be concluded as a result of the present investigation, that all parts of the small intestine of the albino rat exhibit alkaline phosphatase activity. The values decrease exponentially from the pylorus to the ileocolic valve. All cellular fractions show phosphatase activity, namely: microsomes = 76%, nuclei = 10.5%, supernatant fluid = 9.4%, and mitochondria = 7.5%, with a specific activity for the fractions of 52, 2.9, 2.6, and 9.6 respectively.

The response of the enzyme in all fractions is similar in respect ₍to temperature, time of incubation, pH of the incubation mixture, and enzyme concentration.

The slight differences observed in the values of the Michaelis constants and the degree of activation by Mg^{++} may be explained on the basis of a difference in the accessibility of the enzyme of the various fractions to the substrate and Mg^{++} respectively.

We may assume that the same alkaline phosphatase is present in all fractions. In view of the fact that the microsomes contain the highest percentage of the enzymic activity, it may be suggested, that the enzyme is produced chiefly by them. It is also possible that during the different steps of homogenization and fractionation, part of the enzyme is liberated from the microsomes and either becomes attached to the mitochondria and nuclei or remains free in the supernatant fluid.

PART II   THE EFFECT OF FASTING

INTRODUCTION

Fasted animals were to be used for the study of the
effect of ingestion of various amino acids on alkaline
phosphatases. Hence, a knowledge of the phosphatase
activity and amino acid concentrations of the intestine
and blood serum of the fasted animal was considered necessary.

It has been well established that levels of rat blood
alkaline phosphatase fall within 16-24 hours of fasting to
1/4 - 1/3 of the original values and stay there during
continued food deprivation (33). The literature, however,
contains conflicting reports on the effect of fasting on the
intestinal enzyme. Madsen (22) found that the intestinal
phosphatase increased slightly with fasting. On the other
hand, Robinson (40) reported that the values of intestinal
phosphatase fell sharply to 67% of normal after two days
and then further to 53% after 6 days of fasting. The same
author found that the response of the enzyme to force-feeding
of olive oil was progressively lessened with longer periods
of fasting and the group fasted for two days, showed the
highest response. Jackson (23) similarly reported that
intestinal phosphatase decreases with fasting, but he did
not deprive his animals of food for more than one night.

An additional reason for the reinvestigation of the
problem was the fact that Madsen (22) as well as Robinson
(24) used only the supernatant fluid of the intestinal

homogenates for phosphatase determinations, while it was
decided to use the total homogenate for the exploration
of the present project. There is the possibility that
fasting may affect the distribution of the enzyme within
the cell, in which case changes in phosphatase activity
of the supernatant fluid will not represent true changes
in the activity of the total homogenate.

The literature about the blood amino acid concentration
of the rat is very scanty. In fact, we have found only one
report so far. Frame et al.(41) gave the following values
as mgm. of $\alpha-NH_2$-nitrogen per 100 ml., for 24 hour fasted
rats: whole blood, 14.3 - 18.2; plasma, 7.72 ± 0.49; serum,
11.33 ± 0.92. No reference for the amino acid concentration
in the first 10 cm. section (from the pylorus on) of the
intestine could be found.

EXPERIMENTAL

It was noted at the beginning of this experiment, that the ranges of the phosphatase activity of the different groups were overlapping. The use of a large number of animals, therefore, was considered necessary and 88 animals were used altogether. Twenty were killed without previous fasting and these represented the "normal" animal i.e. the animal under standard laboratory conditions, fed Purina fox checkers and water ad libitum. Eight rats were fasted for 1 day, 8 others for 2 days, 14 more for 3 days and 38 for 4 days. Each animal was weighed and housed in an individual cage with plenty of water until the designated time, when it was reweighed and killed by decapitation. The normal animals were killed right after the first weighing. The blood and the first 10 cm. of the small intestine were taken and examined as described in section I.

RESULTS AND DISCUSSION

The results are tabulated in Table VIII and they are graphically represented in Figures 8 and 9 .

Table VIII

Effect of Fasting on the Levels of the Alkaline Phosphatase and Amino Acid Concentrations of Blood Serum and Intestine

Period of fast	Initial body weight (gm)	Weight loss (gm)	Weight of first 10 cm. of intestine (gm)	Alkaline Phosphatase Units/100 gm. of wet intestine	Amino Acids Mgm. α-NH$_2$-N/100 gms. of wet intestine	Mgm. α-NH$_2$-N/100 ml. of serum
0 days	319 +4*	0	0.71 +0.02*	24,800 ± 700*(20)** [175 ± 5]x	96.9 ± 1.9*(18)** [2.60 ± 0.06]xx	8.1 ± 0.3*(16)** [0.77x10^{-4}M]xx
1 day	317 +5	25 +2	0.68 +0.02	21,300 ± 100(8) [144 ± 8]	98.3 ± 3.1 (8) [2.52 ± 0.07]	7.2 ± 0.2 (4) [0.68x10^{-4}M]
2 days	321 +6	35 +2	0.57 + 0.02	21,000 ± 800 (8.) [118 ± 7]	106.7 ± 2.8 (8) [2.31 ±]	6.7 ± 0.1 (4) [0.64x10^{-4}M]
3 days	327 +5	50 +2	0.55 +0.01	21,800 ± 800 (14) [119 ± 4]	102.2 ± 3.1 (14) [2.13 ± 0.07]	7.6 ± 0.3 (10) [0.72x10^{-4}M]
4 days	316 +3	56 +2	0.51 +0.01	23,300 ± 600 (38) [118 ± 4]	107.9 ± 2.5 (34) [2.07 ± 0.06]	7.9 ± 0.2 (28) [0.75x10^{-4}M]

* Standard error of mean
** Number of rats used in each group
x Figures in brackets give units Phosphatase for first 10 cm. intestine
xx Figures in brackets give range molarity of α-NH$_2$-N per hydrolysis mixture during phosphatase determination.
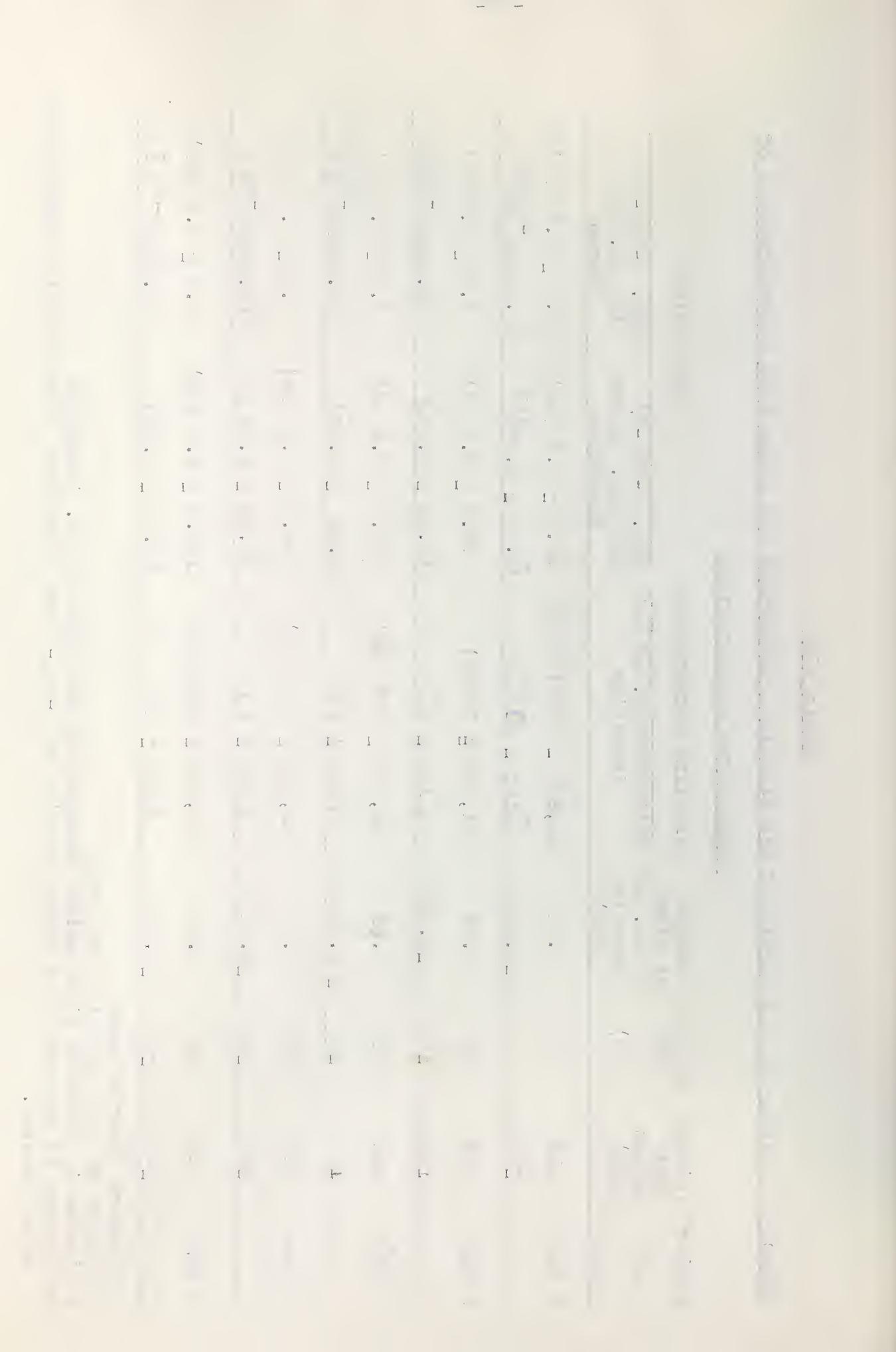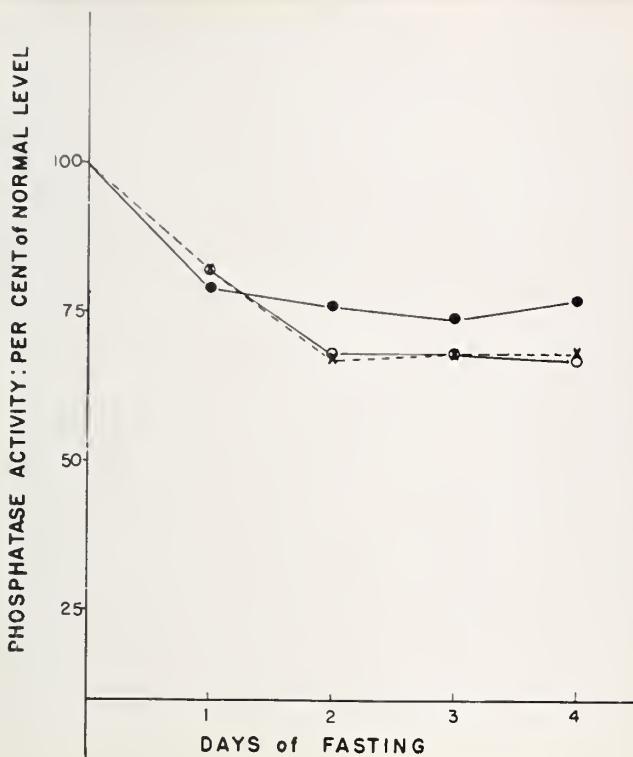
Figure 8.   Effect of fasting on the levels
of Intestinal Alkaline Phosphatase.

(x---x--)= units of phosphatase per 10 first cm. wet
intestine.

(o——o)= units of phosphatase per 100 gms. of wet
intestine corrected for weight losses.

(•——•)= units of phosphatase per 100 gm. initial
body weight.

(1) INTESTINAL PHOSPHATASE

The differences between the mean values of the various groups are not statistically significant when the results are expressed on a weight basis. On the other hand, a highly significant drop in the enzyme activity is detected during the first two days of fasting, if the results are expressed as units per first 10 cm. of intestine.

The intestinal enzyme levels drop to about 82% of the normal value after one day and to 67% after two days. From there on, phosphatase activity remains unchanged. This inconsistency between the two ways of expressing the results is not a real one. If we correct for the weight losses of the intestine during fasting, we can obtain exactly the same information on a weight basis:

Days of Fasting	Units of Phosphatase/ 100 gm. wet intestine (corrected for weight loss)	Percentage of normal values
1	20,400	82
2	16,900	68
3	16,900	68
4	16,700	67

Robinson (24) expressed her results in terms of initial body weight, which is another way of correction for weight losses. Total weight losses, however, do not strictly parallel intestinal weight losses (see Figure 9). In addition it is difficult to weigh rats accurately because they do not stand still during weighing. If we correct the
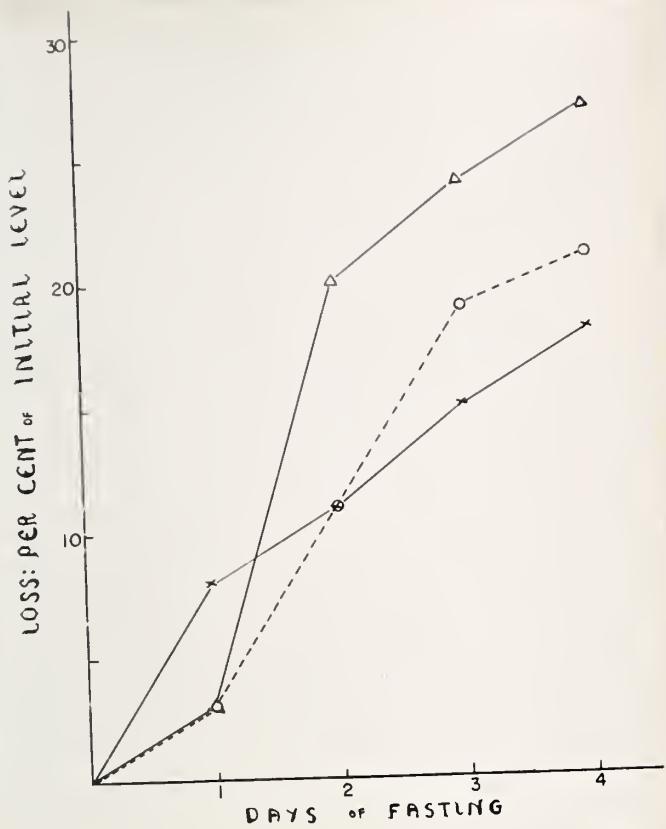
Figure 9. Effect of fasting on body weight
(—x—x), weight of first intestinal section (△—△),
and intestinal amino acid concentration (-○----).

results obtained in the present experiment for initial body
weight, the values become:

1 day of fasting   19,600 units   i.e. 79% of normal value

2 days of fasting   18,800 units   76% of normal value

3 days of fasting   18,400 units   74% of normal value

4 days of fasting   19,200 units   77% of normal value

This way of correction is good enough to show the trend
(see Figure 8).

In the light of the above observation, it is now easy
to realize that Madsen's (22) results for fasted animals
are not at all contradictory with those of Robinson (24).
Madsen expressed his results on an uncorrected weight basis,
while Robinson reported her findings on the basis of initial
body weight. Madsen's values will become considerably lower
after correction for weight losses. He fasted his animals
for a whole week and the weight losses must be quite
substantial for such a long time. A rise above normal levels
may be expected, because the animal continues to lose weight,
while the phosphatase activity after the second day remains
constant.

SERUM ALKALINE PHOSPHATASE

This test was performed on half of the normal rats and
on most of those subjected to a four day fast. A drop to
(27.3 $\pm$ 2 units/100 ml.) 36% of the normal value (76.0 $\pm$ 5.5
units/100 ml.) was noted, which is in good agreement with the
results of previous investigators.

TOTAL AMINO ACID CONCENTRATION IN THE INTESTINAL TISSUE

Here again two ways of calculation have been used:
(a) mgm. of α-NH$_2$-Nitrogen on a weight basis according
to the custom of expressing concentration in tissues.
(b) molar concentration X 10^{-6} of α-NH$_2$-Nitrogen per
hydrolysis mixture. It has been shown that the different
amino acids may act as activators or inhibitors of
alkaline phosphatase depending on their molar concentration
in the hydrolysis mixture. Concentrations between 1 x 10^{-4}
and 1 x 10^{-2} accelerate the action of the enzymes, while
higher concentrations are inhibitory (61). Thus an
accurate knowledge of the molar amino acid concentration
in each case is pertinent. After correction of the first
set of results in Table VIII above for weight losses, we
obtain the following new values:

	mgm. of α-NH$_2$-N/100 gm. of wet intestine (corrected for weight losses)	% of the normal value
1 day of fasting	94	97
2 days of fasting	86	89
3 days of fasting	79	81
4 days of fasting	77	79

The percentages of the molar concentrations of the
different groups in Table VIII are essentially the same,
namely: 97, 89, 82 and 80 per cent for the 4 successive
days of fasting.

Conclusion: A gradual decrease of the total amino acid
concentration in the first 10 cm. of intestine is noted

during fasting. This parallels roughly the weight loss
of the intestine during the same time (see Figure 9).
All values reported here are lower than those required
for activation or inactivation of the alkaline phosphatase.

SERUM AMINO ACID CONCENTRATION

The differences between the mean values of the five
groups are not statistically significant. It seems that
the rat, under normal conditions, has a very effective
mechanism for regulation of the amount of circulating
amino acids. Although there is a continuous absorption
of amino acids from the gut during digestion, their
uptake by the tissues for protein syntheses and their
deamination by the liver are quite rapid, so that normal
values are not exceeded in general. It cannot be
excluded though, that there might be a short rise in the
amino acid concentration of the serum at the peak of the
absorptive phase of the gut, especially after a meal rich
in protein. The normal rats used in the experiments, ate
the standard well-balanced laboratory diet of Purina
fox checkers ad libitum, and so it was not possible to
know when exactly each rat consumed his food, in order to
be able to kill it at the peak of the absorptive state.
At any rate, if such a rise exists, it must be slight and
of short duration, because there was always food present
in larger or smaller amounts in the stomach of the normal
animals at the time of death, and this did not seem to be
reflected in increased amino acid levels.

The values for the 24 hours fasted animals reported here are considerably lower than those found by Frame et al. (41) but no reason can be given for the disparity in results. These authors do not give any information about the storage of sera on which they made their determinations. Some protein breakdown on prolonged standing may occur, even when the serum is kept in the refrigerator. It is necessary to keep it frozen and even then not for more than 10-12 days.

Conclusion: It seems that animals fasted for 3 days are the most suitable for the feeding experiments: the intestinal alkaline phosphatase has been stabilized at its lowest level and the animals do not yet manifest serious effects from the continued food deprivation.

SUMMARY

1. Intestinal alkaline phosphatase activity fell to 82% as a result of a one-day fast and to 67% after a two-day fast. No further change in activity was noted until the fifth day.

2. Sera of animals fasted for 4 days exhibited about 1/3 of the normal levels of alkaline phosphatase.

3. The amino acid concentration of the first 10 cm. of the small intestine was reduced progressively to 79% of normal values in 4 days. This gradual fall paralleled roughly the weight loss of the intestine during the same time.

4. There was no significant change in the amino acid concentration of the serum during fasting.

PART III   EFFECT OF AMINO ACID INGESTION

INTRODUCTION

Wilson and Lewis (74) in 1929, studying the rates of absorption of several amino acids by the gastrointestinal tract of albino rats, found that D-alanine was absorbed slightly faster than the DL-form. Later, Chase and Lewis (71) detected no difference between the absorption rates of L-,D-, and DL-forms of some other amino acids and they concluded that passive diffusion was responsible for the process. This view was supported by the work of Bolton and Wright (72), with cats, and Lathe (73) with dogs. Höber and Höber (75), on the other hand, pointed out in 1937 that diffusion alone could not explain the absorption rates of amino acids by rat intestinal loops and that active processes must be involved. Later several other investigators demonstrated both _in vivo_ (76-79, 108) and _in vitro_ (80-85) that the L-forms of several amino acids are absorbed by the intestine of various animals (rats, dogs, guinea pigs, and hamsters) faster than the D-isomers, against a concentration gradient, and that this absorption was found to be inhibited by anaerobiosis (84), dinitrophenol (82,84), deoxypyridoxine (84), cyanide (82) and tourniquet shock (86).

Cantor, Wight, and Tuba in 1948 (33) demonstrated that high-protein diet produced partial restoration of the low plasma phosphatase activity of fasted animals. Flock and Bollman (19), during the same year, noted that the alkaline phosphatase activity of the intestinal lymph of the rat increased after ingestion of either fat or a fat-free meal. Lawrie and Yudkin (21) in 1949 and Jackson in 1952 (23) reported similar

effects on blood and intestinal alkaline phosphatase produced
by ingestion of a high-protein diet. Tuba and Dickie (26)
noted significant rises in intestinal alkaline phosphatase
activity after ingestion of casein and vitellin.

Recently, Shishova (87, 109) observed that greater amounts of
α-NH$_2$-Nitrogen were absorbed by the intestine of guinea pigs and rats
when inorganic phosphate and adenosine triphosphate were given
with a mixture of amino acids than when the amino acid mixture
was given alone.

From the review of the literature we can assume that
(a) the absorption of some amino acids is an active process
which requires energy and (b) alkaline phosphatase may
participate in this process.

THE EXPERIMENTAL ANIMAL

Adult male albino rats of the Wistar strain, which weighed
260-350 gm. (in most cases, 300-320 gm.) were used in all
experiments. They were housed in individual wide-mesh metal
cages and were fasted for 3 days prior to each experiment.
Water ad libitum was always available. In the morning of the
fourth day they were force-fed 2 ml. of a 0.76M solution of the
amino acid under investigation. In the afternoon at about
5 o'clock the force-feeding was repeated with 3 ml. of the same
solution. Two more feedings of 3 ml. each were given to the
animals during the next day at an interval of 2-4 hours depending
on the rate of absorption of the amino acid tested.

This technique was adopted after considerable experimentation.
Various concentrations (0.125-2.0 M) in varying volumes of

solution (3-5 ml.) were tried. In the beginning a single dose
was given to the animals, which were killed at various times
afterwards (15 minutes, 30 minutes, 1 hour, 3 hours, 5 hours).
The results were very irregular and this may have been due to
                that
the fact∧the animals became excited and nervous during the
force-feeding.

Feeding ad libitum was tried next. Amino acids themselves
are not particularly palatable. Unfortunately fats (17-19, 21-24)
and carbohydrates (16,25) have been reported as affecting
phosphatase levels; so there was not much choice of a carrier
for amino acids which would appeal to the animals. The pure
amino acids were mixed with cellulose, sucaryl (a substitute
for sucrose) and/or apple juice. The combination of flavours
met with little success. The food consumption was very low,
especially in the case of methionine. The addition of sucrose
to individual amino acids did not increase the food intake
appreciably. In addition, in the cases when the consumption
was considerable (2-3 gm.), it was not the same for all animals.
Another drawback of this method was that all the animals did
not eat their rations at the same time. Of course, they had
all been fasted for the same length of time before food was
offered but there was quite a variation in appetites. Force-
feeding seemed once more the method of choice.

It was noted that the rats became excited and nervous only
in the first force-feeding. They were much calmer at the second
one. At the third and fourth they were very docile and in most
cases they actually gave the impression that they liked to be

force-fed. So the first 2 force-feedings were considered

necessary to~the rat with the procedure. The third force-
        accustom

feeding did not produce a marked effect on the levels of the

enzyme. If more than the usual 3 ml. dose was given at one

feeding it was sometimes hard on the animals. Therefore it

was decided that it would be more efficient to give two

separate doses of 3 ml. each at 2-4 hours intervals, depending

on the rate of absorption of individual amino acids. This way

there was a quantity of amino acid continously available for

absorption from the time of the third force-feeding until the

death of the animal. Solutions of amino acids which are 0.76M

are hypertonic. The rats appeared to be uncomfortable following

the feeding of some of the amino acids (lysine, arginine,

tryptophan). In other instances, as with tyrosine, glycine,

leucine, and cystine, the amino acid was very easily absorbed

or eliminated. The stomach was empty after 2 to 3 hours and

the animals seemed comfortable. An ordinary syringe of 5 ml.

was used for the force-feeding. When a soluble amino acid

was force-fed, a narrow catheter 1.5 mm. wide was attached to

the syringe. In the case of an insoluble amino acid, the

catheter was replaced by stomach tubing 3 mm. in diameter.

Note - aqueous solutions or suspensions of the amino acids~used
                                                          were
in all instances.

Monoamino-monocarboxylic acids

Glycine

L-Serine   Soluble. Easy to force-feed.

L-Threonine

L-Leucine: very insoluble. Crystals flaky, adherent. Syringe

and rubber tube (were) often occluded by crystals. It was

necessary to grind the crystals in a mortar before

attempting to dissolve them and to fill the syringe

with the suspension from the back. By rotating the

syringe and tube, crystals were prevented from

adhering to the syringe and force-feeding became

easier. It was (difficult) to evaluate exactly how much of this

amino acid reached the stomach of each animal.

Sulfur containing amino acids

L-Cystine: very insoluble. Crystals heavy, which precipitated

quickly and blocked the mouth of the syringe and

tube. It was necessary to use a less concentrated

suspension (0.57M) and to increase the dosage to

4 ml. The same technique as with leucine was used

in force-feeding. The animals developed slight

diarrhea in most of these experiments.

L-Methionine: very insoluble. Crystals flaky, very light, and

water repellent. Force-feeding was as difficult as

with leucine and cystine.

Monoamino-dicarboxylic acids

L-Glutamic.acid: a large amount of base (1.5 ml. of 5N NaOH

per 10 ml. of suspension) was required in order to

adjust the pH to 7. Completely soluble at this pH.

On post mortem the stomachs showed slight congestion.

The lower parts of the intestine were full of fluid.
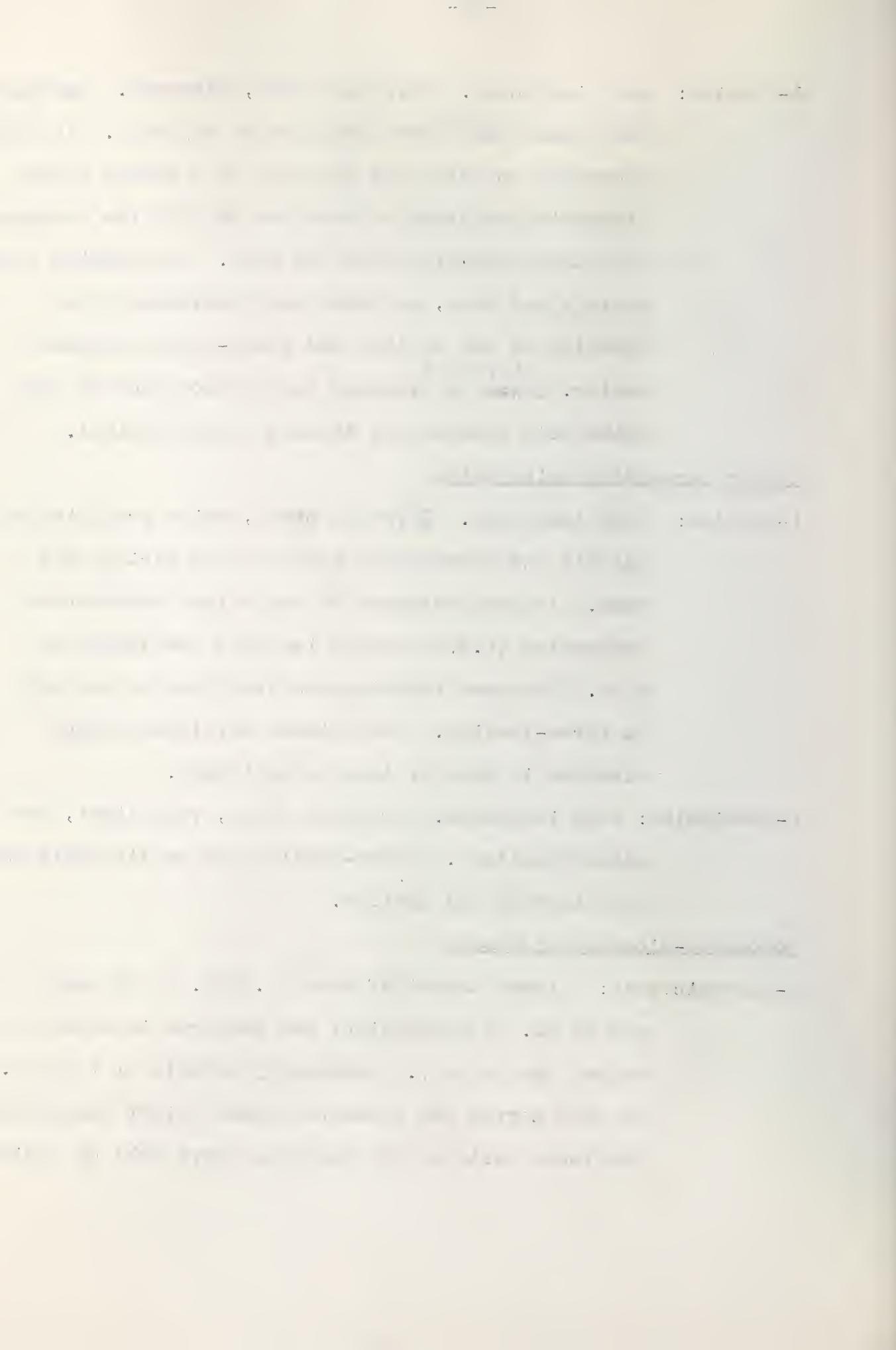
Diamino-monocarboxylic acids

L-Lysine-HCl ⎫ very soluble. No difficulties in force-feeding.

L-Arginine ⎭ All animals developed diarrhea. The entire

gastrointestinal tract was full of fluid at

the time of death.

L-Histidine: not very soluble. Crystals settled down easily.

Aromatic amino acids

L-Phenylalanine: insoluble. Crystals flaky and sticky. Slight

pleasant aromatic odor. Same difficulties as

with methionine.

L-Tyrosine: insoluble. It became necessary to reduce the

concentration to 0.57M and to increase the dosage

to 4 ml.

Heterocyclic amino acid

L-Tryptophan: insoluble. Concentration reduced to 0.57M with

corresponding increase in dosage. Animals

develop diarrhea. On post mortem, although the

stomachs were full of fluid, the duodena seemed

empty. Animals uncomfortable after feeding.

The pH of all solutions or suspensions was adjusted to 7,
except in the case of the glycine-glutamic acid-histidine
mixture. It was found in a preliminary experiment that pH 7
mixtures were absorbed much slower than mixtures of pH 3.9.
It seems that the large amounts of NaOH required to bring this
mixture to pH 7 produced an additional burden on the absorptive
mechanism of the rat. It may be possible that sodium ions are
actively absorbed thus requiring additional work by the intestine.
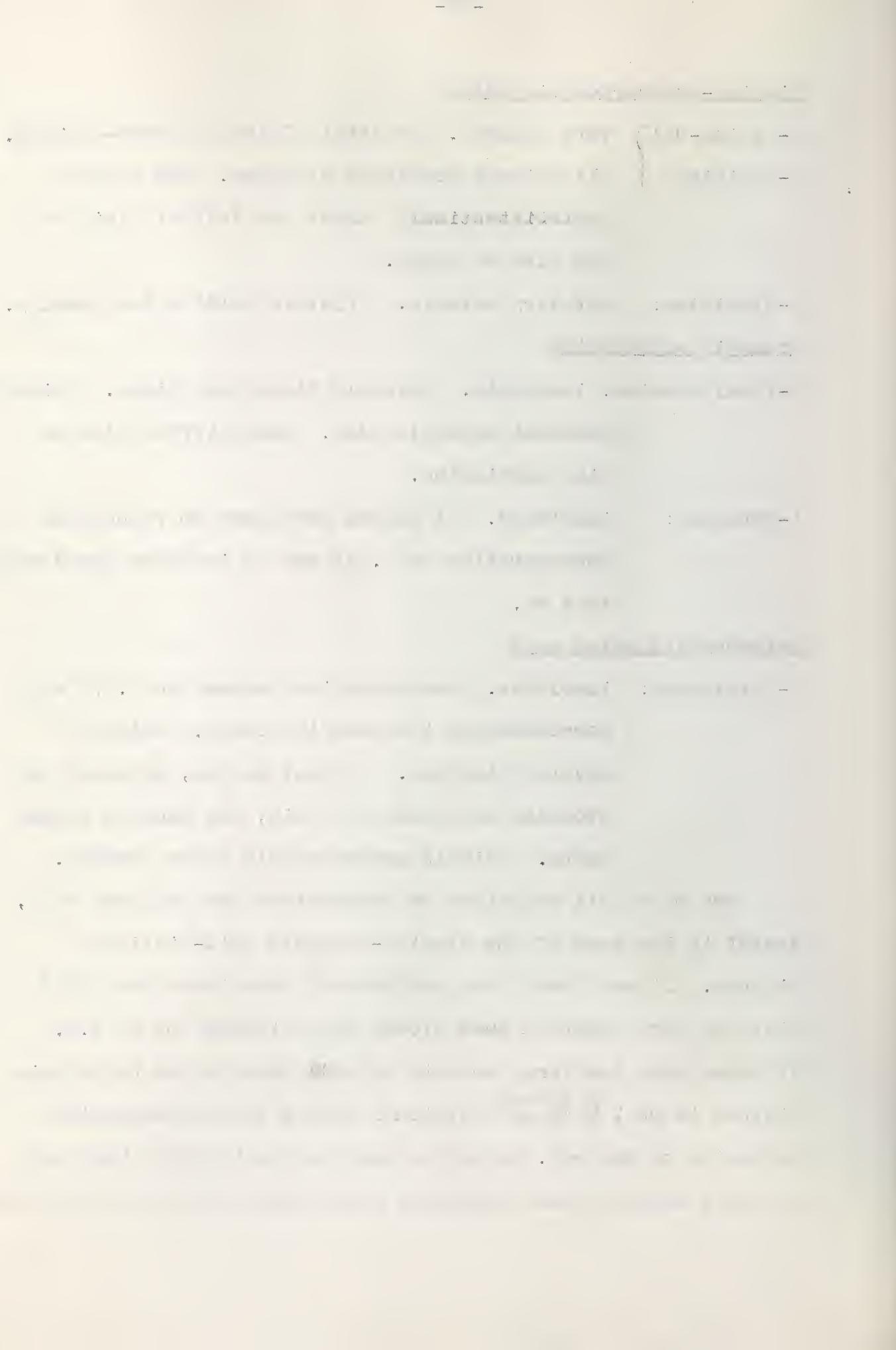
The animals were killed always 2 hours after the last force-feeding, except in the case of the first and third groups which received the above mentioned mixture, when they were killed 3 to 4 hours after.

All amino acids used in these experiments were obtained from Nutritional Biochemicals Corp.

RESULTS

The results are presented in Table IX, X and Figures 10, 11. Table XI shows the statistical analysis of the same results.

Phosphatases and Serum Inorganic Phosphorus

It is evident that all the monoamino-monocarboxylic acids tested produced a significant rise in intestinal alkaline phosphatase and a significant decrease in blood inorganic phosphorus. Among them, only glycine increased the activity of the blood enzyme as well.

Among the sulfur containing amino acids, L-cystine was without any significant effect but L-methionine produced a highly significant elevation of intestinal phosphatase and a highly significant decrease in serum phosphatase and serum inorganic phosphorus.

L-Glutamic acid acted like glycine and increased both the intestinal and serum phosphatases while it decreased serum inorganic phosphorus.

Of the basic amino acids, L-lysine-HCl was without effect throughout. L-Arginine increased the intestinal enzyme without

Table IX

Effect of Amino Acid Ingestion on Levels of Serum and Intestinal Alkaline Phosphatase, Serum

Inorganic Phosphorus and Serum and Intestinal Amino Acid

Amino Acid Fed	Initial body weight (gm)	Wt. loss (gm)	Duode-nal pH	Stom-ach pH	Phosphatase units Intest. /gm	Phosphatase units Serum /100 ml.	Pi Serum mgm/100 ml	Amino Acids as mgm. Intestine Mgm/100 ml.	Amino Acids as mgm. Intestine Molarity/hydr. mix. x10⁻⁶	Amino Acids as mgm. α-NH₂-N Serum Mgm/100 ml	Amino Acids as mgm. α-NH₂-N Serum Molarity/hydr. mix. x10⁻⁶
Contr- ols	316(38) ±3	55.7 ±1.6	6.57	1.96	232(38) ±6*	27.3(30)** ±1.2	7.61x ±0.2	107.9 ±2.5(34)	2.07x ±0.06	7.9(28) ±0.2	7.51x ±0.01
Gly- cine	308 ±5	50.8 ±2.7	5.46	2.21	292(10) ±9	34.7(10) ±2.3	6.4 ±0.2	124.9 ±5.0(14)	2.56 ±0.14	9.2(6) ±0.1	8.76 ±0.01
L-leu- cine	315 ±4	57.1 ±1.0	4.81	2.44	324(14) ±14	27.8(6) ±3.4	6.2 ±0.3	132.0 ±10(14)	2.59 ±0.18	10.6(6) ±0.7	9.52 ±0.07
L-ser- ine	314 ±3	57.1 ±1.7	4.98	2.41	290(14) ±8	31.7(10) ±2.3	5.8 ±0.2	122.8 ±7.3(14)	2.51 ±0.14	9.2(6) ±0.4	8.76 ±0.04
L-thre- onine	311 ±4	56.4 ±0.2	4.22	2.37	270(17) ±10	29.9(10) ±2.2	5.7 ±0.3	119.8 ±5.4(16)	2.33 ±0.07	18.4(10) ±1.2	17.5 ±0.01
L-cys- tine	314 ±3	55.7 ±1.8	6.73	1.73	249(14) ±12	24.2(10) ±3.3	7.4 ±0.2	126.6 ±4.2(13)	2.36 ±0.07	8.4(9) ±0.3	8.00 ±0.03
L-meth- ionine	317 ±5	58.5 ±2.3	3.66	2.86	297(14) ±8	15.9(10) ±1.6	4.9 ±0.2	129.3 ±8.6(14)	2.39 ±0.13	23.5(6) ±0.3	22.4 ±0.02
L-glu- tamic	314 ±5	52.5 ±2.2	6.47	5.45	291(13) ±11	37.1(10) ±1.7	5.9 ±0.3	132.8 ±5.1(13)	2.56 ±0.12	11.2(6) ±0.5	16.67 ±0.04
L-ly- sine	317 ±3	51.3 ±1.7	3.54	2.61	229(12) ±9	25.7(4) ±3.6	7.1 ±0.6	121.4 ±5.3(12)	2.51 ±0.10	8.8(4) ±0.4	8.38 ±0.04
L-arg- inine	316 ±4	41.1 ±1.6	5.31	3.68	301(13) ±14	26.8(6) ±2.5	7.7 ±0.3	122.8 ±7.0(13)	2.49 ±0.14	6.5(6) ±0.2	6.13 ±0.01
L-his- tidine	316 ±4	56.8 ±1.8	5.42	3.03	215(14) ±11	34.0(10) ±3.5	6.1 ±0.2	139.1 ±5.5(13)	2.61 ±0.69	20.5(6) ±2.4	19.5 ±0.30
L-phen- ylal.	314 ±5	52.8 ±1.7	4.54	2.91	244(16) ±10	24.8(8) ±2.5	5.7 ±0.04	140.4 ±5.6(14)	2.79 ±0.11	16.7(8) ±1.3	15.9 ±0.2
L-tyro- sine	312 ±7	54.5 ±2.5	6.89	1.77	223(14) ±7	27.3(8) ±3.1	6.2 ±0.2	120.2 ±6.4(12)	2.38 ±0.14	9.9(8) ±0.4	9.42 ±0.03
L-tryp- tophan	318 ±4	57.3 ±1.9	5.70	2.90	201(10) ±6	30.2(10) ±3.3	5.1 ±0.3	104.1 ±6.7(10)	2.00 ±0.13	9.1(6) ±0.9	8.66 ±0.09

* Standard error of mean.

** Number of rats per group.

x Number of rats in each group of this column is same as in preceding one.
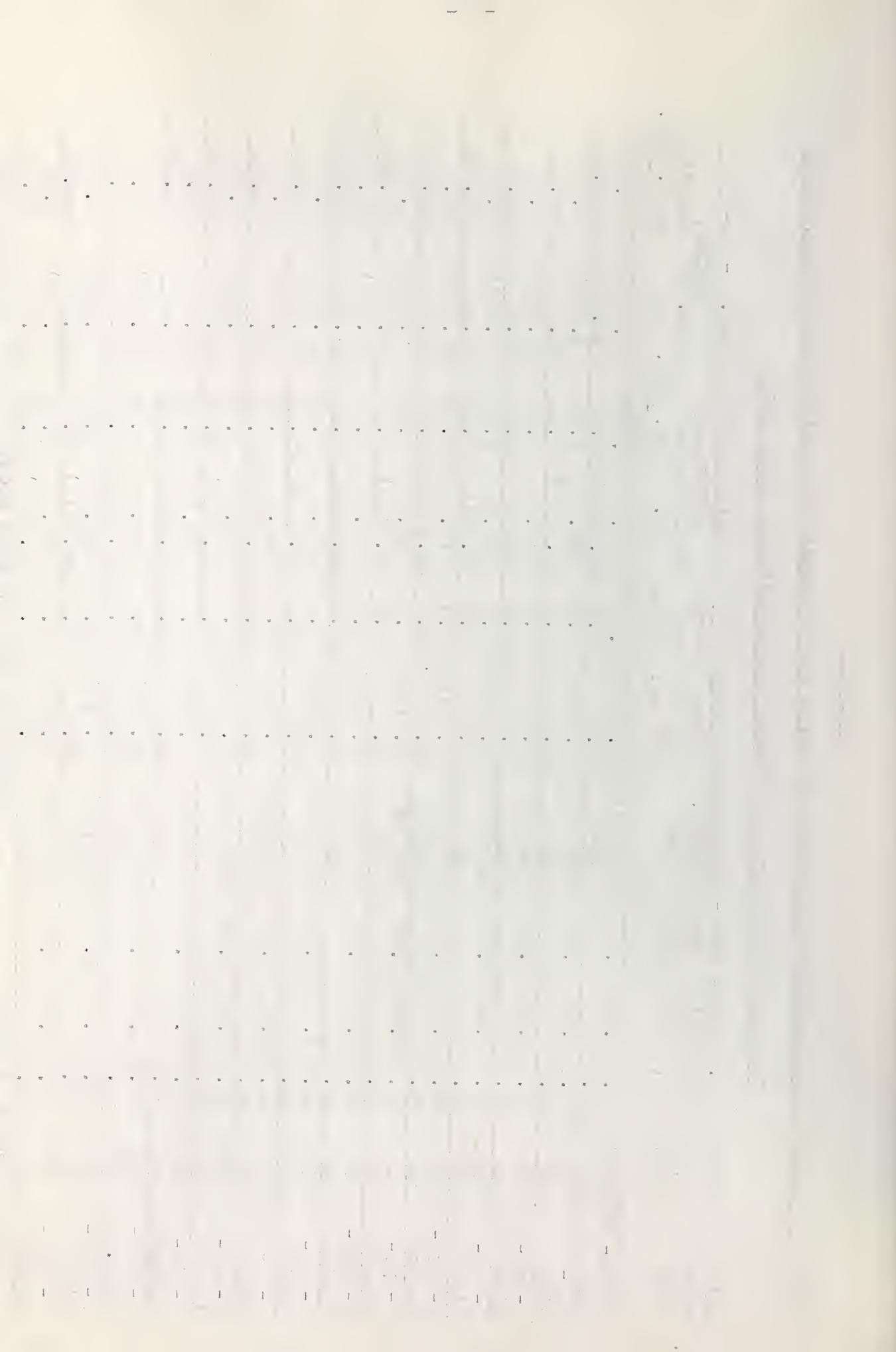
Table X

Effect of Force-feeding a Mixture of Glycine, Glutamic Acid and Histidine on Levels of Serum and Intestinal Alkaline Phosphatase, Serum Inorganic Phosphorus, and Serum and Intestinal Amino Acids

Amount of mixture force-fed	Phosphatase		Pi	Amino Acids			
				Intestine		Serum	
	Intestine units/gm.	Serum units/100ml	Serum mgm/100ml	mgm α-NH$_2$-N/ 100 gms.	Molarity/ hydr. mix. x10^{-6}	mgm α-NH$_2$-N/ 100 gms.	Molarity/ hydr. mix. x10^{-6}
0.76M Glutamic, 0.76M Glycine, 0.76M Histidine Fed 4 times	315+16* (7)**	47.6+8.2 (7)	6.4+0.6 (7)	141.5+5.6 (6)	2.78+0.16 (6)	29.5+4.9 (6)	28.1
G - G - H (each 0.25M) Fed 4 times	232+13 (6)	41.4+1.2 (6)	7.6+0.2 (6)	109.9+4.4 (6)	2.21+0.56 (6)	17.1+0.8 (6)	16.3
G -G - H (each 0.76M) Fed 3 times	242+13 (6)	34.3+2.6 (6)	7.2+0.2 (6)	119.2+5.6 (6)	2.68+0.14 (6)	13.2+2.1 (5)	12.6

* Standard error of mean
** Number of rats per group.

Table XI

Statistical Analysis of Data in Tables IX and X (Values of P)

Amino Acids	Phosphatase		P_i	Amino Acids	
	Intest.	Serum	Serum	Intest.	Serum
Glycine	<0.01	<0.01	<0.01	<0.01	<0.01
L-leucine	<0.01		<0.01	<0.05	<0.01
L-serine	<0.01	>0.05	<0.01	>0.05	<0.02
L-threonine	<0.01	>0.30	<0.01	>0.05	
L-cystine	>0.20	>0.30	>0.30	<0.01	>0.20
L-methionine	<0.01	<0.01	<0.01	<0.05	
L-glutamic acid	<0.01	<0.01	<0.01	<0.01	<0.01
L-lysine	>0.50	>0.50	>0.40	<0.05	<0.05
L-arginine	<0.01	>0.50	>0.10	>0.05	<0.01
L-histidine	>0.10	<0.01	<0.01	<0.01	
L-phenylal.	>0.20	>0.30	<0.01	<0.01	
L-tyrosine	>0.30		<0.01	>0.05	<0.01
L-tryptophan	<0.01	>0.40	<0.01	>0.50	>0.20
Glutamic + Glycine + Histidine (each 0.76M) Fed 4 times	<0.01		>0.05	<0.01	<0.01
G - G - H (each 0.25M) Fed 4 times	>0.50	<0.01	>0.10	>0.50	
G - G - H (each 0.76M) Fed 3 times	>0.50	<0.02	>0.10	>0.05	<0.03
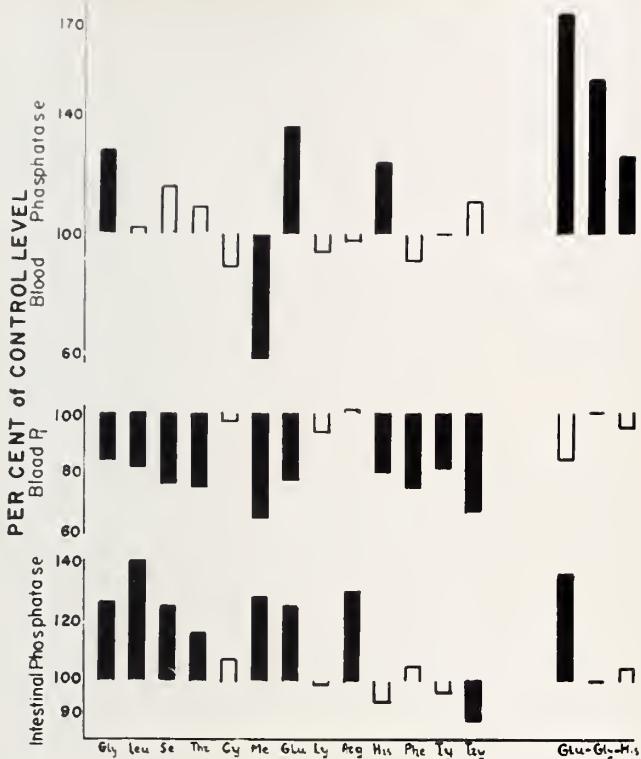
Figure 10. Effect of ingestion of each of various amino acid solutions on the levels of Intestinal Phosphatase, Serum P_1, and Serum Phosphatase. Black bars=statistically highly significant change ($P < 0.01$). Empty bars=statistically insignificant results ($P > 0.05$).
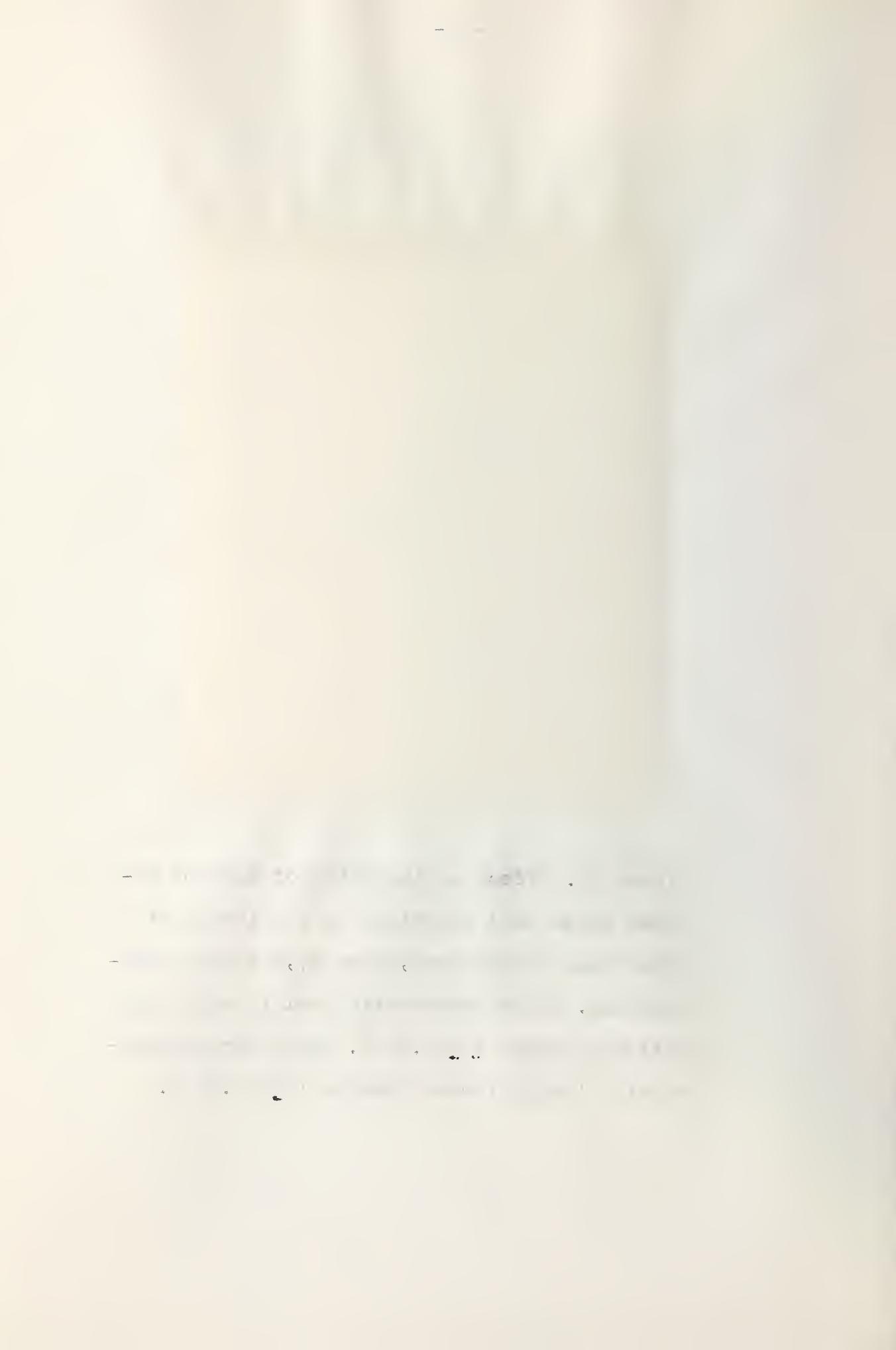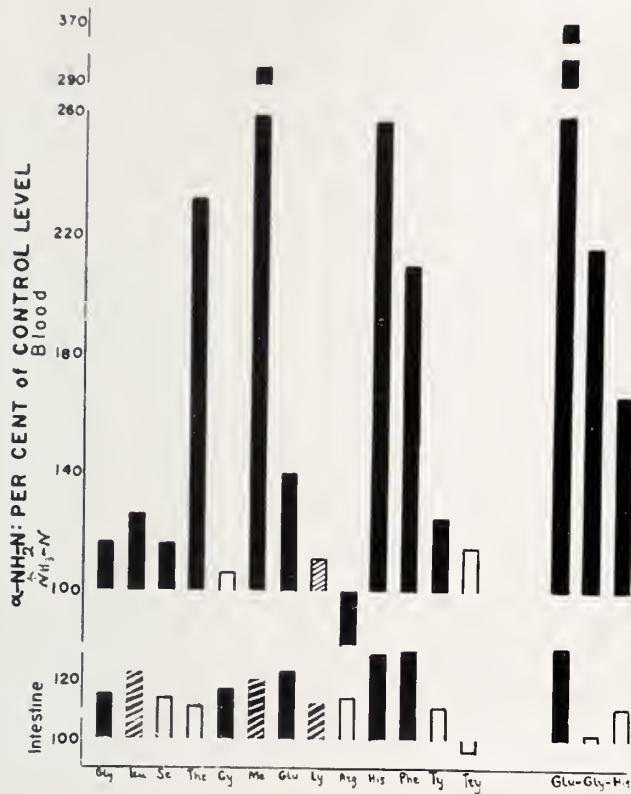
Figure 11. Effect of ingestion of each of several
amino acids on α-NH₂-N and NH₃-N concentration of
Serum and Intestine. Black bars=statistically high-
ly significant results: ($\underline{P} < 0.01$). Hatched bars=
=statistically significant results ($\underline{P} < 0.05$).
White bars=statistically insignificant results
($\underline{P} > 0.05$).

affecting the serum enzyme or inorganic phosphorus. L-Histidine had no effect on the intestinal phosphatase, but it elevated serum phosphatase and decreased serum inorganic phosphorus values.

L-Tryptophan decreased significantly the activity of the intestinal alkaline phosphatase and the concentration of blood inorganic phosphorus.

When glycine-L-glutamic acid-L-histidine mixture was force-fed twice on the last day and in a high concentration (0.76+0.76+0.76M), it produced a highly significant elevation of both enzymes, but only the serum enzyme was affected when the concentration of the mixture was reduced to 0.25+0.25+0.25M or when the number of force-feedings was cut down to 3 (i.e. one during the last day of the experiment). Serum inorganic phosphorus was unaffected in all 3 groups.

AMINO ACIDS

Intestine: The concentration of α-NH$_2$-Nitrogen was increased in most instances. L-Serine, L-threonine, L-arginine, L-tyrosine, L-tryptophan, were the exceptions as well as glycine-L-glutamic acid-L-histidine mixture, given in low concentrations or with only one force-feeding during the last day.

Serum: L-Cystine and L-tryptophan did not show any effect. All the other amino acids increased the total α-NH$_2$-Nitrogen concentration of the serum with the exception of L-arginine which produced a highly significant drop.

DISCUSSION

Generally alkaline phosphatase is considered almost exclusively as a hydrolytic enzyme. Any increase in its activity
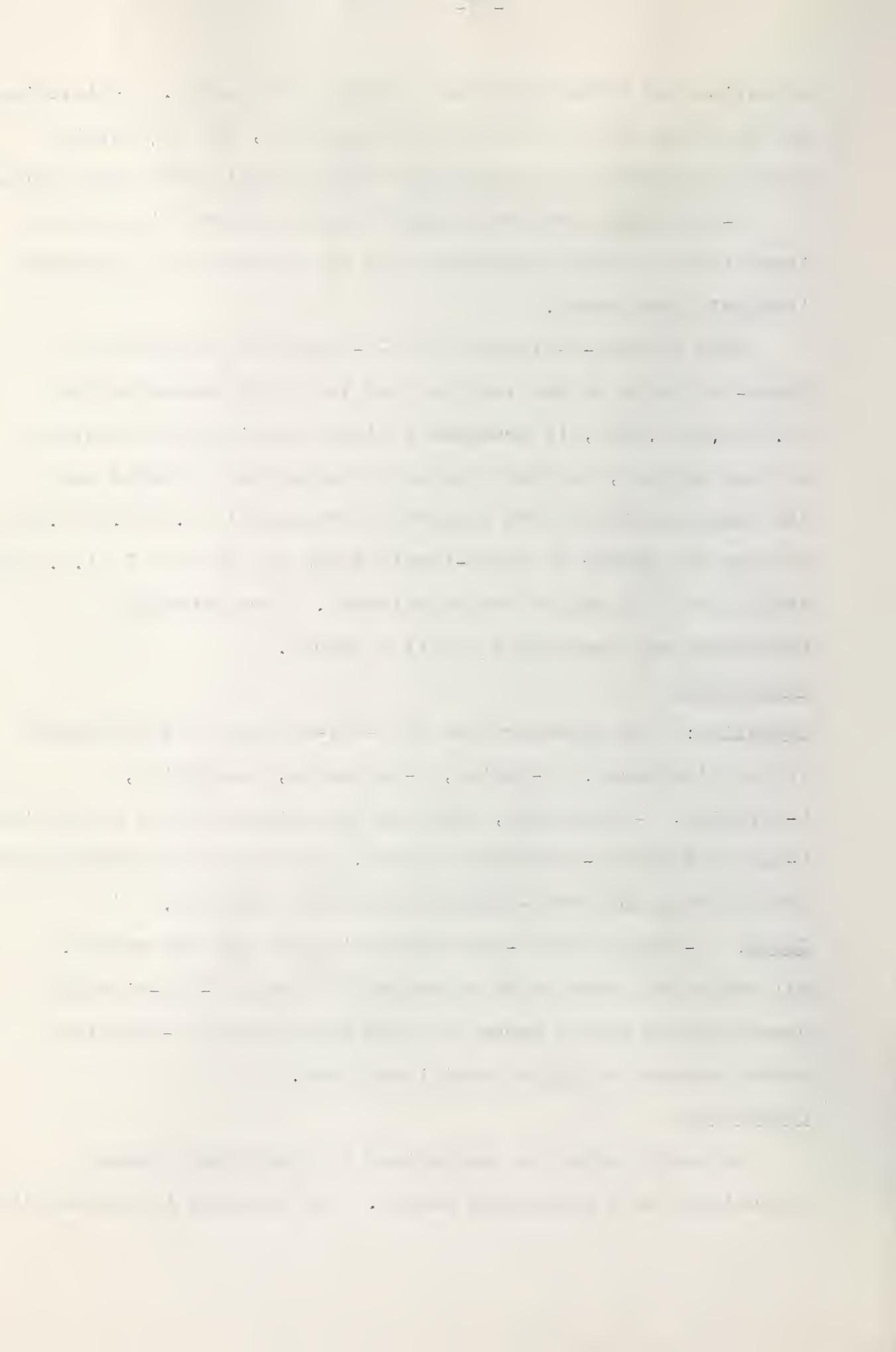
in the body is usually interpreted as a result of increased
hydrolysis of phosphomonoesters. According to this view
one may interpret the results of the present experiment in
the following way.

The seven amino acids which elevated the activity of the
intestinal alkaline phosphatase are phosphorylated by the
action of a phosphokinase-Adenosine Triphosphate (ATP) system at
the surface of the intestinal epithelium, transferred through
the cells in the phosphorylated form and then they are
dephosphorylated by alkaline phosphatase before they enter
into the portal blood. If this were the case there should be
either an increase in inorganic phosphorus concentration by
the amount of phosphorus liberated through this hydrolytic
action or, if we assume that this phosphorus is reutilized
for the synthesis of new ATP, there should be no change in
inorganic phosphorus. This did not happen here. Actually
the opposite was observed i.e. inorganic phosphate uptake
in all cases except those of arginine and the glycine-glutamic
acid-histidine mixture where no significant change in serum
inorganic phosphorus was noted.

It has long been established by histological methods
(54,55,56,57) that the alkaline phosphatase is located in the
striated border of the intestinal epithelium. This striated
border forms the end of the cell which faces the intestinal
lumen. If the only role of phosphatase in the intestine were the
hydrolysis of phosphomonoesters then its location would be ideal
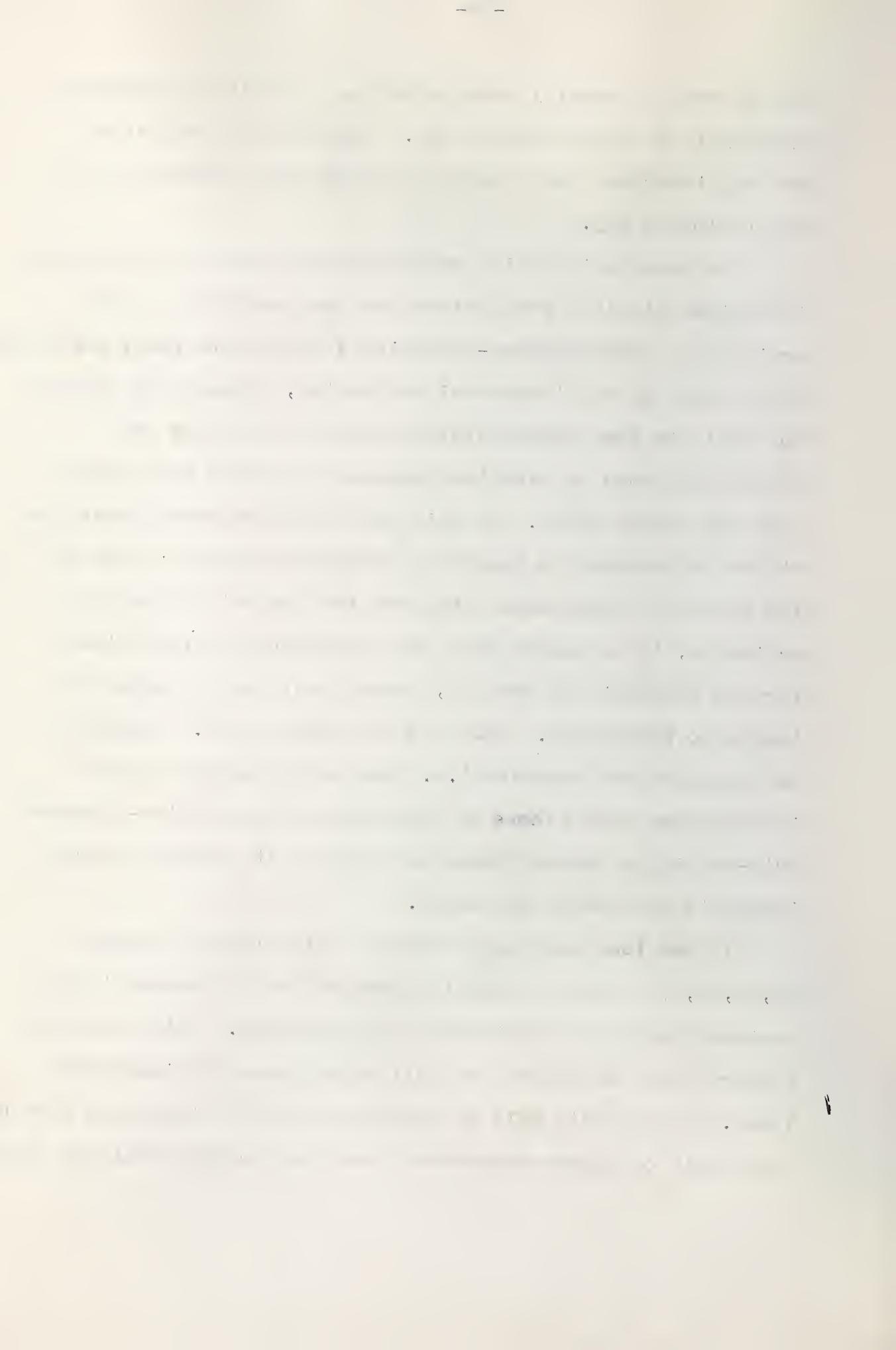
for the hydrolysis of esters which enter the body preformed i.e. already phosphorylated. But as far as the theory of "absorptive" phosphorylation is concerned the alkaline phosphatase is located in the wrong end of the cell. As soon as the sugar, lipid, amino acid, whatever the case may be, becomes phosphorylated by an ATP-phosphokinase system it would be dephosphorylated by alkaline phosphatase at once before it had the chance to get into the cell.

Decrease in the concentration of inorganic phosphate of the serum may be produced by either an increased excretion of phosphorus by the kidney or by phosphorus uptake by the tissues and synthesis of organic phosphates. There are no obvious reasons to believe that the first possibility may be involved in our experiments.

The synthetic activity of crude phosphatase preparations from various organs has been demonstrated by Martland and Robison in 1927 (6), Kay (7) in 1928 and recently by Green and Meyerhof (9) with purified preparations. Evidence for the transphosphorylating ability of phosphatase has also been presented by the last authors (12,13,14) as well as by Axelrod (10,11) and Morton (15). According to Green and Meyerhof the transfer of phosphate by means of phosphatase seems to be a common phenomenon. Morton found that all substrates which are hydrolysed by the enzyme i.e. true orthophosphomonoesters, phosphoamides, phosphoenol pyruvate and adenosine-5-phosphate, can act as donors for the transferase reaction but not ATP or ADP (ADP is Adenosine Diphosphate). Green and Meyerhof think

that ATP may participate in this reaction but to a smaller
extent than the other phosphate donors do.

Morton postulates that the enzyme combines with the
phosphate donor as follows:

$$E + PR \longrightarrow EPR \longrightarrow EP + ROH$$

where E = phosphatase

PR = phosphate donor

EPR = phosphatase-phosphate donor complex

EP = phosphorylated enzyme (activated)

ROH = dephosphorylated phosphate donor

The activated enzyme contains a reactive phosphorus probably
bound to the phenolic OH of a tyrosine residue. Morton says
further that there seems to be no good reason for attributing
transferase activity any longer to enzymes associated with,
but distinct from hydrolases.

In the light of this information one is tempted to explain
the findings of the present experiment in the following manner.
Glycine, L-leucine, L-serine, L-threonine, L-methionine, and
L-glutamic acid become phosphorylated by alkaline phosphatase
as soon as they enter the intestinal cells. The overall
reaction may be represented as follows:

(1) amino acid + P_1 $\xrightarrow{\text{Phosphatase}}$ amino acid - P.
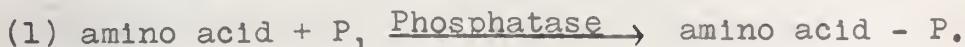
By means of this phosphorylation the concentration of the
free amino acid within the cells falls to zero, so the
concentration gradient between cell and intestinal fluids is
kept high and consequently more and more amino acid enters into
the cell. As a result, the concentration of the phosphorylated
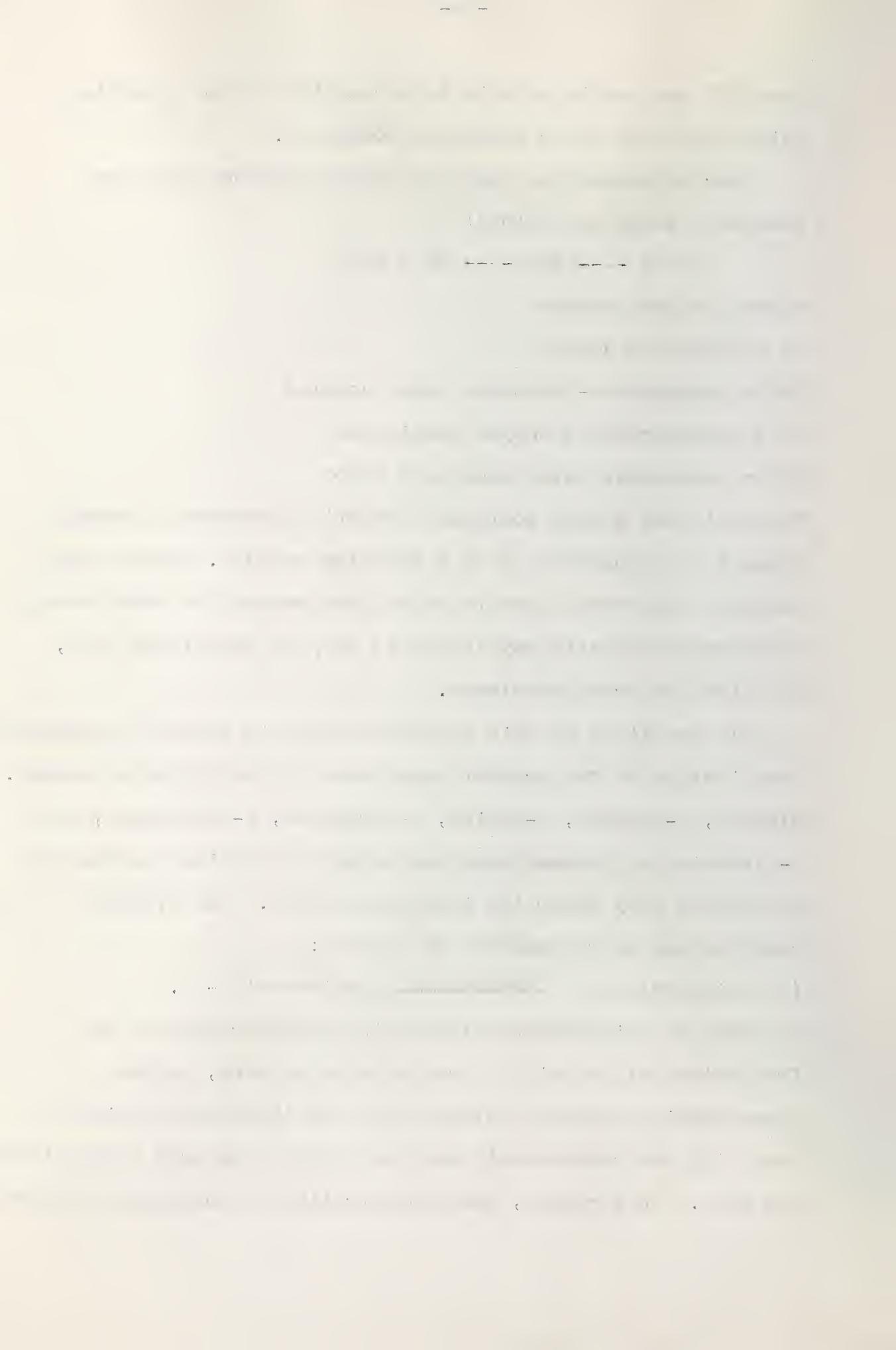
amino acid increases continuously and the compound starts to move by diffusion towards the opposite side of the cell. In this place there is no free amino acid present so far (or very little). The dynamics of the reaction (1) are now different, and favor the reverse direction.

(2) amino acid-P $\xrightarrow{\text{Phosphatase}}$ amino acid + P_i

The free amino acid starts accumulating at this end until a concentration gradient between the cell and the subepithelial capillaries is developed. Then the amino acid passes into the blood stream and is eliminated from the site of the reaction. Thus the concentration gradient is always kept high and the whole process goes on until there is no more free amino acid in the gut. Certainly, the velocity slows down gradually as the tissues become saturated with the amino acid and the blood amino acid concentration goes up.

For the maintenance of this mechanism sufficient amounts of phosphatase and inorganic phosphate are required. Phosphatase levels go up at the expense of other tissue proteins and inorganic phosphorus is drawn from the blood after the cell's stores are exhausted.

Glycine and glutamic acid increased the levels of the blood enzyme as well. A probable explanation could be that in these cases the affinity of phosphatase for glycine and glutamic acid is stronger than for the other amino acids. The enzyme-substrate complexes require a longer period of time to dissociate and thus they may be transferred into the blood before they have had enough time to dissociate.

The opposite effect, i.e., a decrease of the level of serum alkaline phosphatase was manifested following the ingestion of methionine. This observation was made previously by Tuba et al.(88). They reported in 1949 that methionine, added to the diet of weanling rats, maintained on low-protein rations, decreased the high serum alkaline phosphatase levels considerably. Tuba and Shaw (89) noted an analogous phenomenon with rats fed a high-fat, low-protein diet. These authors found that there should be a definite ratio between fat and methionine in order to maintain the phosphatase levels at the normal value.

This phosphatase depressing effect of methionine can be explained, if we take into consideration that in all the above mentioned cases there is always more or less a degree of liver damage. Starvation, prolonged administration of a low-protein, high-fat diet are classical methods for producing fatty livers in laboratory animals. It is well known clinically that some liver disorders (29) (obstructive and hepatocellular jaundice, cases of portal cirrhosis etc.) are associated with elevated alkaline serum phosphatase. This has been observed in laboratory animals also. Hough and coworkers (90,91) reported that liver damage produced in dogs by protein deficient diet was accompanied by high serum alkaline phosphatase levels. Both these changes could be prevented by the administration of choline. Methionine, by furnishing methyl groups for the synthesis of choline, improves the liver condition and consequently reduces the elevated serum phosphatase.

By the same reasoning, we can explain why Tuba and Madsen (92) did not find any significant depression of serum phosphatase due to dietary methionine itself. These authors were working with animals maintained on Purina fox checkers, a well balanced laboratory diet. There was no liver damage, and no abnormal serum phosphatase activity which methionine could return to normal.

Along with the previously postulated phosphorylation mechanism, transphosphorylation reactions catalysed also by phosphatase may well take place. Meyerhof and Green (12) have shown that both mechanisms can operate simultaneously in the presence of purified alkaline phosphatase _in vitro_.

Arginine feeding yielded no evidence of inorganic phosphorus uptake. Transphosphorylation reactions involving phosphopyruvate, possibly ATP or other organic phosphate donors may explain the observed increase in intestinal alkaline phosphatase activity.

The increased serum phosphatase activity in the group fed histidine is not likely of intestinal origin. The simultaneous decrease in inorganic phosphate indicates that phosphorylation took place elsewhere in the body. It has been demonstrated by a number of investigators (93-96) that the "microsomes" of several tissues incorporate labelled amino acids at a much faster rate (about 3 times) than do other cell elements. "Microsomes" are particularly rich in alkaline phosphatase. The elevation of the levels of the blood enzyme observed after feeding histidine may be considered as an indication of the

participation of the enzyme in the incorporation of histidine into microsomal proteins. It could be suggested that the intestinal enzyme has been translocated into the serum. Absorption was not completely over at the time of the death of the animals. There was still some amount of solution present in the stomach. If the increased serum phosphatase activity was of intestinal origin there should be still some elevation of the intestinal phosphatase levels as in the case of glycine and glutamic acid.

Tryptophan caused a significant decrease of the activity of the intestinal enzyme, accompanied by a decrease in blood inorganic phosphorus. It is hard to explain these changes. It is of course known that amino acids may act as inhibitors of alkaline phosphatase but the concentrations required for this effect are relatively high, from 1×10^{-3} M and over (61,62,97). The mean concentration of tryptophan in the experiments reported here was only 2×10^{-6} M, which is even less than that of the control animals. There is certainly the possibility that large amounts of this amino acid were already bound to the enzyme and this could not be quantitatively determined. Such an inhibition may explain the slow absorption of the tryptophan. It hardly raised the serum amino acid concentration above fasting levels. Greenstein and coworkers (98) have studied the toxicity of several amino acids and they found that tryptophan was the most toxic to rats. The rats used in our experiments seemed very uncomfortable after the last force-feeding.

The decrease in inorganic phosphorus may be due to a compensatory action of other enzymes e.g. stimulation of glycolysis and oxidative phosphorylation.

The glycine-glutamic acid-histidine mixture resulted in a highly significant elevation of serum alkaline phosphatase values. This is not surprising in view of the fact that feeding the mixture had the same effect as the individual amino acids when they were given singly to three groups. The effect on inorganic phosphorus and intestinal phosphatase was quite different. It seems that phosphorylation was inhibited in all three groups while transphosphorylation was inhibited only in the last two. Antagonism between amino acids has been reported by several authors. Elvejhem and coworkers (99) found that leucine was antagonistic to isoleucine and valine; threonine was antagonistic to phenylalanine and tyrosine. Pinsky and Geiger (100) noted that tryptophan interferes with the absorption of L-histidine. Kamin and Handler (101) published results of experiments with rats (_in vivo_) in which it was shown that invariably the presence of an excess of an amino acid delayed the absorption of another amino acid; thus the rate of absorption of glutamic acid was inhibited by histidine to 41% and by glycine to 64%; that of histidine was retarded 50% by glutamic acid and 63% by glycine.

The lack of any activating effect on the intestinal alkaline phosphatase in the last two groups may be simply due to the fact that glycine and glutamic acid which were given in smaller amounts, were already absorbed by the time of the death of the animal and thus the excess phosphatase was already transferred to the blood.

The only effect manifested by cystine was an increase of the intestinal α-NH$_2$-N concentration, which may well be due to adherence of particles to the villi and incomplete elimination by washing. This amino acid is very insoluble. Its rate of absorption seemed very slow. It did not even raise the serum α-NH$_2$-N level. Of course, it cannot be excluded that it is not a slow absorption rate which is responsible for this phenomenon but that the rate of incorporation or elimination of this amino acid is equal to the rate of absorption so no accumulation in the serum occurs.

Although lysine-HCl is very soluble, it barely raised the serum and intestinal α-NH$_2$-N concentration to levels of significance. The rats fed this amino acid suffered from diarrhea. This could result in reduced absorption. However in the case of arginine, where diarrhea also developed significant effects on intestinal phosphatase and serum α-NH$_2$-N concentration were observed.

Both tyrosine and phenylalanine produced a highly significant rise in the levels of the serum α-NH$_2$-N concentration accompanied by inorganic phosphorus uptake but none of these amino acids seemed to affect the alkaline phosphatases. It is probable that both stimulated phosphorylation but the non-specific phosphatases did not seem to have participated in it.

As it was mentioned earlier (61,62,97) amino acids may behave as activators or inhibitors of intestinal alkaline phosphatase. In concentrations of 1 x 10^{-4} to 1 x 10^{-2} M per hydrolysis mixture they accelerate the action of the enzyme,

while in higher concentrations they manifest an inhibitory effect. The levels of α-NH_2-N in the intestinal homogenates obtained in our experiments were all below (about 50 times) activation concentration. Of course one cannot exclude the possibility that considerable amounts of amino acids were already combined with the enzyme thus activating or inactivating it. In this case the elevation of intestinal alkaline phosphatase activity may be considered as a phenomenon analogous to the in vitro activation observed by Bodansky without any implication of phosphorylation or transphosphorylation. In such an instance the uptake of inorganic phosphorus must be attributed to the action of other enzymes. It cannot be excluded however that both ~~the~~ mechanisms may be implicated in the experiments reported by us. The rise in α-NH_2-N concentration of the intestinal homogenates cannot be considered always as increased concentration of amino acids within the intestinal cells themselves. It is possible, especially in the case of insoluble amino acids, that some particles were adherent between the villi and could not be washed out.

The serum concentration of free α-NH_2-N in the experiments with threonine, methionine, histidine, phenylalanine and the glycine-glutamic acid-histidine mixture were in the range where activation should be expected, if the serum enzyme shows the same sensitivity towards the amino acids as intestinal, bone, and kidney phosphatases do. Of these, only histidine and the three-amino-acid mixture, increased serum phosphatase activity

significantly. No significant effect was exerted by phenylalanine or threonine while a decrease in phosphatase activity was produced by methionine. Of course not all the amount of the recorded α-NH_2-N was really amino acid nitrogen. Ammonia also reacts with napthoquinone. The amount of ammonia in the serum of fasted and normal animals is negligible but considerable amounts may be produced after ingestion of large quantities of amino acids.

The elevation of serum α-NH_2-N concentration is the result of two factors: (a) prolonged absorption from the gut and (b) inability of the tissues and especially of the liver to get rid of the excess. Arginine produced a highly significant decrease in α-amino-N concentration. This is not surprising. The catalytic effect of arginine on the urea cycle is very well known after the classical work of Krebs and Henseleit (102). Arginine keeps the cycle going on. The excess ammonia produced by the deamination of the ingested amino acids is eliminated. The arginine reserves of the control animals must have been exhausted, and moreover a slight degree of liver damage (fatty infiltration), due to starvation, must be present. As a result the α-NH_2-N concentration of the serum of control rats was higher than that of arginine-fed animals. This action of arginine is the reason for the beneficial effect this amino acid exerted during toxicity experiments with lethal doses of mixtures of other amino acids (103).

Statistical comparison of the values for intestinal and serum phosphatases and serum inorganic phosphorus, obtained after force-feeding of leucine and of threonine showed a highly significant difference between the intestinal phosphatase values. This may possibly indicate a difference in the mechanism of absorption of these amino acids or may simply be due to a difference in their absorption rates. Threonine is almost completely soluble at the concentration of 0.76M, while leucine is very insoluble. It is possible that at the time of death i.e. two hours after the last force-feeding, the absorption of leucine and consequently the stimulation of intestinal alkaline phosphatase activity due to this amino acid was still optimal, while the effect of threonine was already on the decline. Table IX, as well as Figures 10 and 11 show that the amount of amino acid present in the intestinal wall at the time of death was higher in the case of leucine. Generally, for the monoaminomonocarboxylic acids differences in intestinal phosphatase activity roughly paralleled differences in intestinal amino acid concentration. This was also true for the glycine-glutamic acid histidine mixture, and tryptophan, but was not so for the remaining amino acids listed in the table. This close correlation, whenever observed, may be considered as an additional indication of the participation of intestinal alkaline phosphatase in amino acid absorption.

The results of the present experiments represent just one chronological phase and a very limited one of the absorption process of some amino acids. This study is restricted to determination of the changes in the levels of intestinal and serum alkaline phosphatases, and intestinal and serum amino acids after 4-7 hours of continuous absorption. This time was selected, because it was thought that 4-7 hours of continuous stimulation would be enough for the enzymes to adapt themselves to the increased demands imposed upon them, if they actually take part in amino acid absorption. Time did not allow us to examine what happens before or after this absorption period. The increase in the levels of the alkaline phosphatases, especially that of intestine, which was observed in some instances may be taken as an indication of the participation of these enzymes in the absorption of these amino acids. Thus it was found once more that intestinal alkaline phosphatase can adapt itself to some food stimuli (22,24,104).

Solutions of NaCl of 0.76M concentration force-fed to 6 rats under the same conditions as the amino acids did not show any significant effect on intestinal and serum alkaline phosphatases.

SUMMARY

Alkaline Phosphatases and Serum Inorganic Phosphorus

The force-feeding to rats of glycine, L-leucine, L-serine, L-threonine, L-glutamic acid and L-methionine increased significantly the levels of intestinal alkaline phosphatase. The speculation was made that this may be a result of phosphorylating and transphosphorylating activity manifested by the nonspecific alkaline phosphatase. Glycine and glutamic acid raised the levels of serum phosphatase as well, while methionine produced a highly significant drop in the enzyme levels. This may be attributed to the improvement of a slight liver deficiency which accompanies starvation.

L-Arginine raised only the intestinal alkaline phosphatase activity. Phosphorylation catalysed by phosphatase could be implicated.

L-Histidine activated only the serum alkaline phosphatase. A possible explanation was discussed.

L-Tryptophan produced a highly significant decrease of the activity of intestinal alkaline phosphatase under the conditions of these experiments. Inactivation of the enzyme by tryptophan could be responsible.

A mixture of glycine-L-glutamic acid-L-histidine produced elevation of serum alkaline phosphatase in all experiments. The intestinal enzyme was affected only when the mixture was given in large amounts. No inorganic phosphorus uptake could be demonstrated although this was observed with every one of the individual amino acids when they were force-fed separately.

α-Amino-Nitrogen

Serum: Cystine and lysine were without effect. All the other amino acids tested increased the α-NH_2-N concentration except arginine which produced a highly significant drop. This was attributed to stimulation of the urea cycle.

Intestine: The α-NH_2-N concentration of intestinal homogenates was increased after force-feeding of glycine, L-leucine, L-cystine, L-methionine, L-glutamic acid, L-lysine-HCl, L-histidine, L-phenylalanine and large amounts of a mixture of glycine-L-glutamic acid and L-histidine.
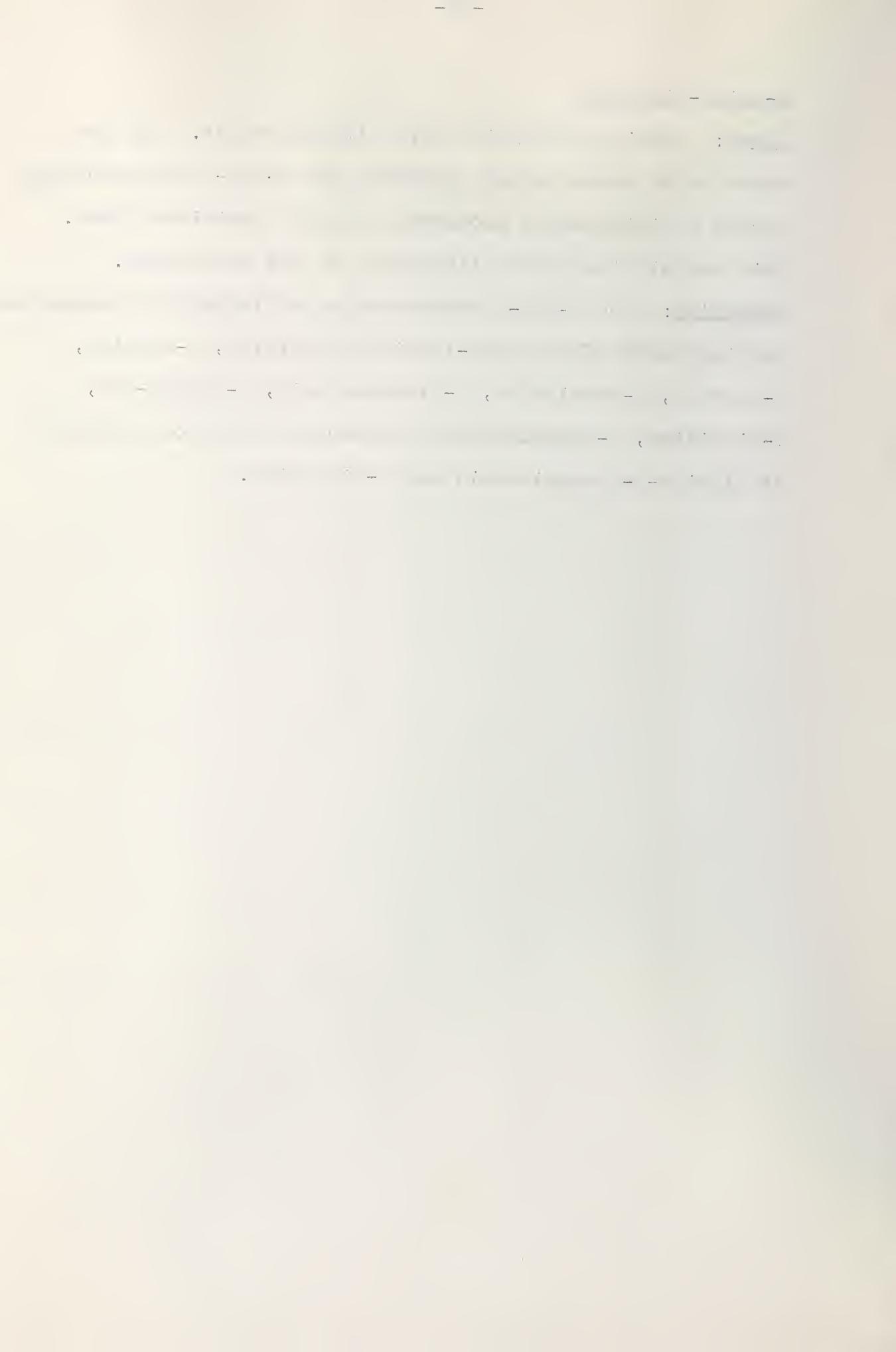
PART IV   EFFECT OF FORCE-FEEDING AMINO ACID SOLUTIONS OF LOW

AND HIGH pH VALUES ON THE INTESTINAL ALKALINE

PHOSPHATASE OF THE ALBINO RAT

Many of the amino acids are barely soluble in water,
e.g. leucine, phenylalanine, methionine, cystine, tyrosine,
tryptophan. Some also have the tendency to stick to the glass
and rubber tubes and this makes force-feeding very difficult.
Besides the occlusion of the syringe and tubes, which happened
frequently in the beginning, even in the uneventful cases a
good number of crystals were left clinging to the glass and
rubber surfaces. It actually seemed impossible to force-feed
some of these amino acids. The solubility on these occasions
is considerably improved by lowering the pH of the solution.
The pH of the stomach of fasted animals is about 2 (see Table IX).
Friedman (105) found that the average volume of gastric juice
secreted during a 30 minute period was 2.0 ml. Therefore, it
was thought that, if 2-3 ml. of solution of pH 2.0 were
introduced into the stomach, carefully avoiding any leakage of
the fluid in the mouth and oesophagus, this would not be harmful
to the animals. It was decided to force-feed 2 rats with such
an acid solution of leucine and see how it was tolerated by the
animals. The method described in Part III was used again except
that in this experiment three force-feedings were used in all
instead of the usual four. The animals did not seem particularly
uncomfortable after the feedings. On post mortem examination the
stomachs and the duodena manifested a normal appearance. We
were surprised to find that the intestinal phosphatase levels

were unusually low (4,100 units/100 gm. wet intestine).

In order to find out whether leucine or the very acid pH

was responsible for this depression, a soluble amino acid

glycine, was force-fed in solutions adjusted to 2 different

pH values (pH 2 and pH 7), to two separate groups of animals.

The results solved the problem unmistakably. The animals fed

the neutral solution exhibited an intestinal alkaline phosphatase

activity about 29,000/100 gm. wet intestine, while those fed

the acid solution had an activity of 2,400 units, i.e. there was

about 92% inhibition.

This preliminary experiment indicated that the problem

was worth further investigation. It was decided to investigate

if (a) other amino acids had the same effect, (b) this

inhibition could be exerted on normal animals as well, (c) one

dosage of 3 ml. of acid solution was enough to produce this

characteristic depression on the levels of the intestinal

phosphatase and (d) alkaline pH values had any analogous effect

on the enzyme.

EXPERIMENTAL

Adult male albino rats as usual were used in these

experiments also. One group of them was prepared and force-fed

as described in Section III. (i.e. They were starved for 3 days,

force-fed twice on the fourth and twice on the fifth day a 0.76M

solution of the amino acid under investigation). Finally they

were killed 2 hours after the last force-feeding. The only

difference was that the solutions given to them were no longer

neutral. Their pH values varied from 2.0 to 11.0. There were

usually two animals for each pH value for each amino acid tested. These experiments were usually carried out at the same time as the effect of neutral solutions of the same amino acid was studied. A second group consisted of non-fasted animals, or rats fasted for a short period (12 hours) in order to make reasonably sure that their stomachs were empty at the time of force-feeding. These animals were given only one dosage of 0.76M solution of methionine of pH 2.0. A third group contained rats fasted for 4 days. These were likewise given only 1 dosage of the acid methionine solution (pH 2.0).

As soon as the peritoneal cavity was exposed a pair of forceps was applied at the pylorus, another pair at the small intestine about 15 cm. below, and a third pair at the cardiac end of the stomach. This was done in order to avoid the mixing of the gastric and duodenal fluids as well as to prevent their loss, because it was decided to determine the pH values of these fluids.

In most cases, and this is especially true for the very alkaline solutions (pH 10.5 and 11.0) there were some hemorrhages in the stomachs, usually small (petechiae), and fewer petechiae in the duodenum. These symptoms were pronounced in the animals force-fed glycine. One of them actually became moribund after the fourth feeding.

The pH values of the gastric and duodenal fluids were determined each time by means of a Beckman one drop electrode, mounted on the Beckman Model G, Portable pH Meter.
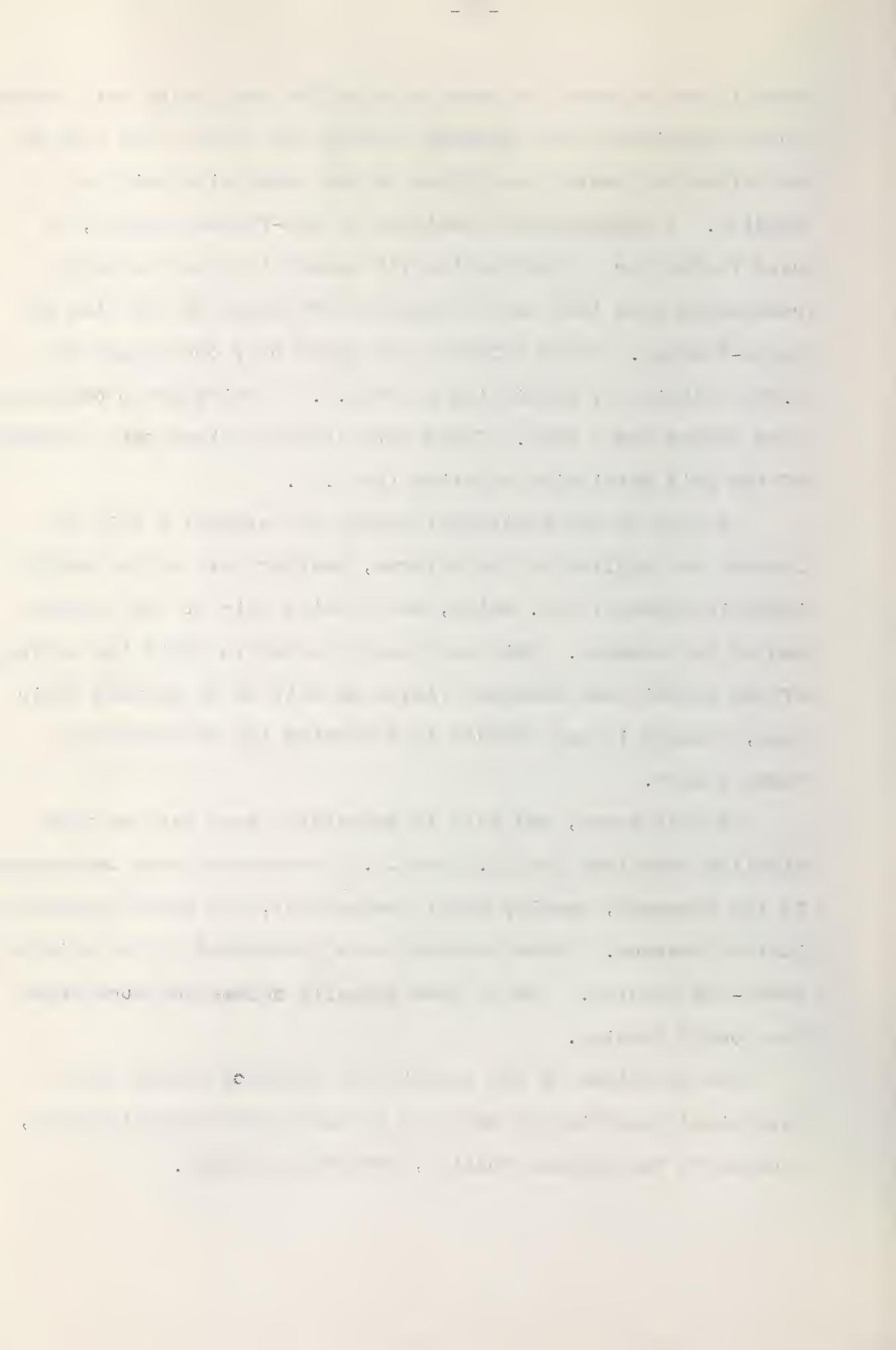
RESULTS:

Group 1

It can easily be seen from Table XII that most amino acids have a profound depressing effect on intestinal alkaline phosphatase when they are given as a solution of pH 2.0. Glycine reduced the activity to 8% of the value observed when this amino acid was given as a neutral solution, methionine to 9%, leucine to 12%, histidine to 14%, lysine to 22%, tryptophan to 23%, and phenylalanine to 27%. On the other hand, glutamic acid had only a slight inhibitory effect. It reduced the activity to 57 and 65%, according to the amount given, while tyrosine was ineffective.

Solutions of amino acids adjusted to alkaline pH values before force-feeding also produced inhibition of the enzyme, although this was not so pronounced as that evoked by acid solutions. i.e. Glycine given at pH 10.5, reduced the activity of the intestinal phosphatase only to 35%, glutamic acid to 73%, phenylalanine to 46%, and arginine to 63%. Only tryptophan manifested a stronger inhibitory action when it was given at pH 11.0 than when it was given at pH 2.0. Tyrosine also was an exception to the rule. It now reduced the enzyme activity to 71% while at pH 2.0 it was without effect.

Groups 2 and 3

All rats inluded in these groups received only 3 ml. of a 0.76M solution of methionine of pH 2.0 (one dosage). The results are tabulated in Table XIII.

In sub-group I are normal rats whose stomachs were more or less filled with Purina fox checkers at the time of force-feeding. The intestinal alkaline phosphatase activity of these rats

Table XII

Effect of pH of 0.76M Solutions of each of Various Amino Acids on the Activity of

Intestinal Alkaline Phosphatase after Repeated Force-feedings

Amino Acid Fed	pH								
	2	3	5	6	7	9	10	10.5	11
Glycine	24*+6** (2)x			254+21 (2)	293 (10)	216+10 (2)	110+8 (2)	104+1 (4)	
L-leucine	41+5 (4)				324 (14)				
L-glutamic acid	189+1xx 167+11 (2)	247+11 (2)			291 (10)	258+3 (2)	211+54 (2)		
L-methionine	27+10 (4)				297 (14)	320+26 (2)	160+2 (2)	144+6 (4)	
L-lysine-HCl	50+18 (2)	259+19 (2)	271+30 (2)		229 (12)				
L-histidine	29+4 xxx (4)				215 (14)				
L-arginine					301 (13)	294+7 (2)	240+40 (2)		190+5 (2)
L-phenylal.	66+22 (2)				244 (16)			112+5 (2)	
L-tyrosine	236+11 (4)				223 (14)			159+5 (2)	
L-tryptophan	46+8 (2)				201 (10)				33+7 (2)

* Intestinal Phosphatase expressed as units/gm. of wet intestine.
** Standard error of mean.
x Number of rats per group.   xx rats given only 2 ml. of acid solution.
xxx Rats received only one force-feeding on last day.

Table XIII

The Effect of One Force-feeding of a 0.76M Solution of
Methionine, pH 2.0, on the Activity of the Alkaline
Phosphatase of the Duodenum of the Albino Rat

| Group | Time after force-feeding | | |
	2 - 3 hours	4 - 5 hours	Controls
I	22,200* ±4,700** (4)*** P>0.5	26,800 +950 (2)	
II	16,300 ±1,200 (4) P<0.01	19,500 +1,400 (2)	24,800 +700 (20)
III	13,100 ±1,700 (3) P<0.01	13,600 ±2,800 (2)	
IV	6,100 +1,700 (4)	10,000 ±3,000	23,200 +580 (38)

* Units of phosphatase per 100 gms.
** Standard error of mean.
*** Number of rats in each group.

All animals received 3 ml. of methionine solution except
group II which received only 2 ml.

was more or less unimpaired depending on the amount of food
they had in the stomach at the time of force-feeding. The
results were statistically insignificant.

In sub-group II were classified rats whose stomachs had
a small amount of food at the time of intubation. Their alka-
line phosphatase activity was lowered very significantly, $P < 0.01$.

Sub-group III contained rats starved for 12 hours prior
to force-feeding. There were still traces of food in their
stomachs. The depression of intestinal alkaline phosphatase
activity was even greater, $P < 0.01$.

In sub-group IV were included rats starved for 4 days prior
to force-feeding. All manifested a pronounced decrease in phos-
phatase activity (1,200-9,000 units/100gm. of wet intestine.)The-
re was no need of statistics to evaluate these results.

It seems that the depressing effect of acid solutions on
the enzymic activity did not last very long. The phosphatase le-
vels of the groups killed 4-5 hours after force-feeding were al-
ways higher than those of the animals killed within 2-3 hours.

The effect of force-feeding acid or alkaline solutions on
the serum alkaline phosphatase and on serum and intestinal ami-
no acid concentration was not so pronounced. The number of ani-
mals in each group was very small (2-4) and statistical ana-
lysis of the results would not be reliable.

DISCUSSION:

Schuchardt (105) noticed in 1936 that certain yeasts were
unable to hydrolyse α-and β-glycerophosphate at alkaline pH levels
if they had been previously suspended in dilute acetic acid,while
they lost their ability to hydrolyse these esters in acid (105)

reaction mixtures if they had been presuspended in NH_4OH.
Tuba (106) made a similar observation with Toronto strain of
Saccharomyces cerevisiae yeast. He suspended the organisms
in buffers at various pH values for 24 hours. After this he
determined acid and alkaline phosphatase activities at the
optimum pH of each enzyme. He found that alkaline glycero-
phosphatase was completely inactivated, if the organisms had
been suspended in buffers of pH 2.8-4.0. Suspension in buffers
of a higher pH were less harmful. The higher the pH, the greater
the residual phosphatase activity until pH 8.6. Beyond this
point a decrease in phosphatase activity was again demonstrated.

From a comparison of the pH values of gastric and duodenal
fluids represented in Table XIV we see that: (a) there was a
very small decrease in the pH values of gastric fluids of
animals fed the pH 2.0 solutions as compared with those of the
animals fed the neutral solutions, except in the case of
glutamic acid, where a lowering by 3 pH units was observed.
(b) The pH values of the duodenal fluids were generally lower
in the animals fed the acid solutions except in the experiments
with tyrosine, where there was no difference between animals
force-fed an acid or a neutral suspension of this amino acid.
(c) Generally the pH values of the gastric and intestinal juices
of the animals force-fed alkaline solutions were higher than
those fed the neutral solutions. The more alkaline the force-
fed solution was, the higher became the pH of the gastric and
intestinal contents. Exceptions were the animals force-fed the
glycine solutions of pH 9.0 whose duodenal fluids had a lower
pH value than those of animals force-fed a neutral solution.

Table XIV

pH Values of Gastric and Duodenal Fluids of Rats Fed 0.76M Solutions of Amino Acids

Adjusted to Various Hydrogen Ion Concentrations

Amino Acid Fed	pH of solution fed									
	2		7		9		10		10.5	
	Duod.	Stom.	Duod.	Stom.	Duod.	Stom.	Duod.	Stom.	Duod.	Stom.
Glycine	3.27 ±0.50*(2)	2.36 ±0.02	5.46 (10)	2.21	4.20 ±1.0(2)	3.75 ±0.40	6.95 ±0.46(2)	4.86 ±1.96	7.50 ±0.30(4)	7.66 ±0.40
L-glutamic acid	4.01 ±0.01(2) 4.25 ±0.21(2)	2.11 ±0.90 2.21 ±0.22	6.47 (10)	5.45	7.43 ±0.11(4)	7.52 ±0.56	8.36 ±0.21 (4)	8.23 ±0.85		
L-methionine	2.88 ±0.30(4)	2.17 ±0.02	3.66 (14)	2.86	7.41 ±0.57(2)	7.83 ±0.05	9.05 ±0.03(2)	9.45 ±0.21	8.98 ±0.04(4)	9.48 ±0.10
L-lysine-HCl	3.27 ±0.50(2)	2.36 ±0.02	3.54 (14)	2.61						
L-arginine			5.31 (13)	3.68	7.61 ±0.12(2)	7.87 ±0.35	6.75 ±0.52(2)	6.76 ±0.56	6.98** ±0.14(2)	5.68** ±1.34
L-phenylal.	2.59 ±0.05(2)	1.93 ±0.20	4.54 (16)	2.91					9.19 ±0.17(2)	9.40 ±0.13
L-tyrosine	6.94 ±0.40(4)	1.87 ±0.10	6.89 (14)	1.77					6.99 ±0.37(2)	5.61 ±0.33
L-tryptophan	4.18 ±0.90(2)	2.24 ±0.04	5.70 (10)	2.90					9.62** ±0.16(2)	9.87** ±0.60

* Standard error of mean.
** Values taken at pH 11.0.
Values in brackets refer to number of rats per group.
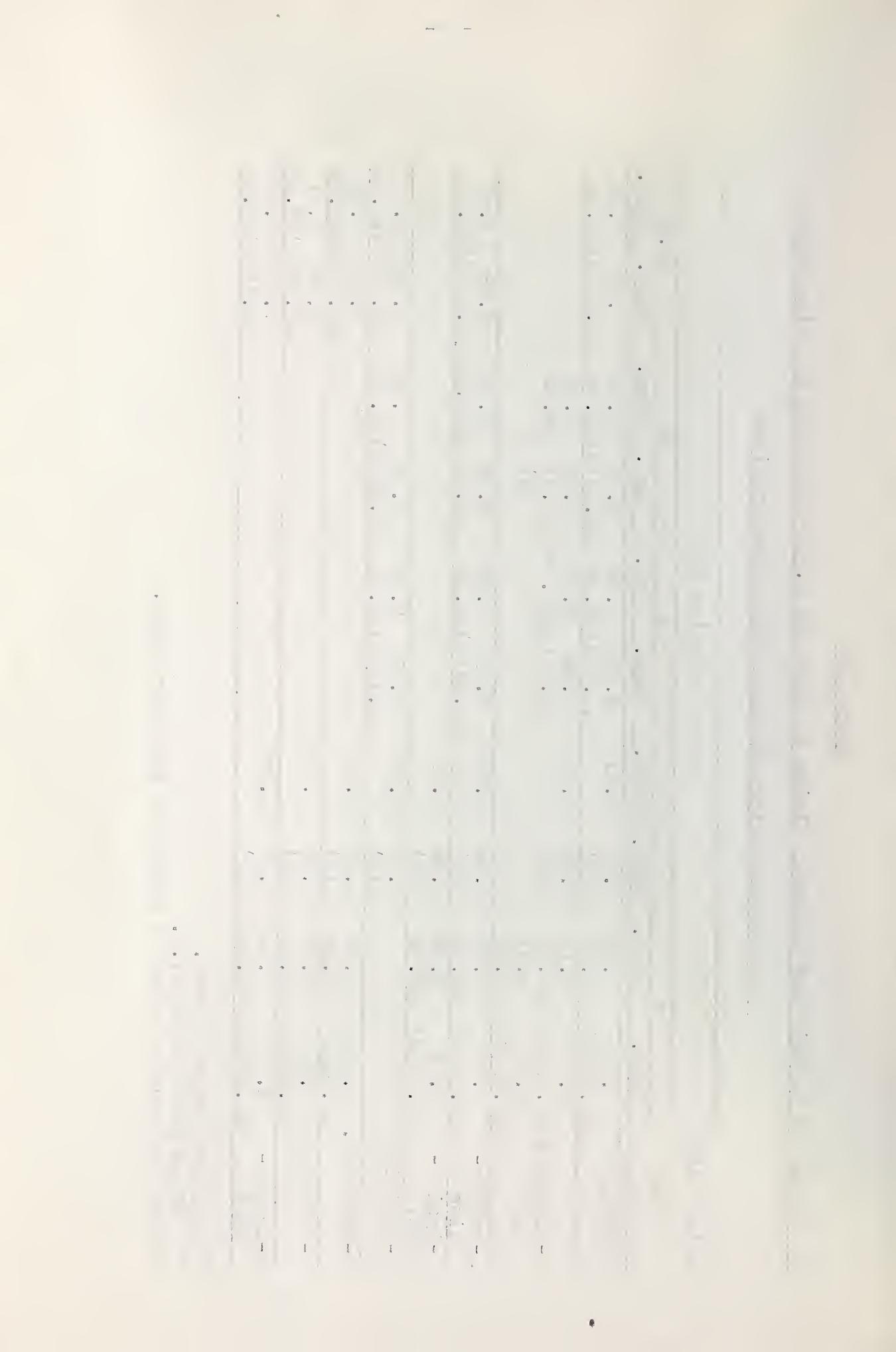
Similarly the gastrointestinal fluids of animals force-fed arginine solutions of pH 10 and 11 exhibited lower pH values than those given a pH 9.0 solution of the same amino acid.

No relationship between pH of gastric and duodenal fluids on the one hand and phosphatase activity on the other, could be detected. This may be due to the fact, that the hydrogen ion concentration of the duodenum was neither steady nor the same throughout its whole length. Each time the pylorus opened and gastric juice poured in, the pH went down momentarily until bile, pancreatic and intestinal secretions could neutralize or at least reduce its acidity. Values listed in Table XIV represent the hydrogen ion concentration of the first drop obtained from the proximal end of the duodenum. The pH of the second drop was always higher.

During the adjustments of the various amino acid solutions to pH 2.0, it was noted that different amounts of HCl acid were required for this purpose. Furthermore, the intestinal alkaline phosphatase activity exhibited by the rats fed these solutions seemed to be proportional to this amount. When the number of milliequivalents of acid given to the animals/dosage together with the amino acid solution, was plotted against the phosphatase activity, the enzyme activity observed subsequent to the feeding (expressed as percentage of the activity of animals fed the same amino acid at the neutral pH), a curvilinear line resulted (Figure 12a). By expressing the results as percentages of the activity at pH 7.0, the specific effect of each individual amino acid on intestinal alkaline phosphatase was

eliminated and consequently the degree of inhibition produced
by the force-feeding of the acid solutions could be better
estimated. When the logarithm instead of the percentage of
activity was plotted, the straight line in Figure 12b was
obtained. If we take into consideration that there is a wide
range of variation in the levels of intestinal alkaline
phosphatase from animal to animal even in the fasted state,
and that each point of the graph represents the results
obtained from 2-4 rats only, we can easily explain the
deviations. When solutions of 0.76M NaCl or distilled water,
both adjusted to pH 2.0 (0.04 meq. of HCl/3 ml. of solution or
water were required), were force-fed to rats, they did not
produce any statistically significant lowering of the intestinal
phosphatase activity as compared with animals force-fed neutral
0.76M solutions of NaCl and neutral water. Statistically
insignificant similarly was the increase in phosphatase activity
produced by the force-feeding of pH 2.0 solutions of tyrosine.
The regression equation calculated from the experimental data
was $\hat{Y} = 0.76x+0.81$ i.e. the log of the percentage of phosphatase
activity decreased at the estimated rate of 0.76 per meq. of
acid under the conditions of these experiments. The use of
larger numbers of animals would evidently give a better
representation of the situation. It would probably change the
regression coefficient a little, or possibly indicate some
irregularities. However, the fact will remain that the decrease
in phosphatase activity produced by force-feeding solutions of

Table XV

Residual Intestinal Alkaline Phosphatase Activity Expressed as % of Value at pH 7, and

Meq. of Acid or Base Used to Adjust the pH of Several Amino Acids at the pH Values Indicated

Amino Acid Fed	pH of solution fed										
	2		9		10		10.5		11	
	%*	Meq.**	%	Meq.	%	Meq.	%	Meq.	%	Meq.
Glycine	8.2	1.38	74	0.39	38	1.34	35	1.87		
L-leucine	12	1.26								
L-glutamic acid	65 57	0.20 0.30	89	0.75	73	1.60				
L-methionine	9.1	1.30	100	0.52	54	1.81	49	2.07		
L-lysine-HCl	22	0.77								
L-arginine			98	0.76	80	1.76			63	2.07
L-phenylal.	27	0.98					46	1.95		
L-tyrosine	100	0.07					71	2.71		
L-tryptophan	23	0.75							16	2.08

* % phosphatase activity left

** Meq. of acid or base per dosage
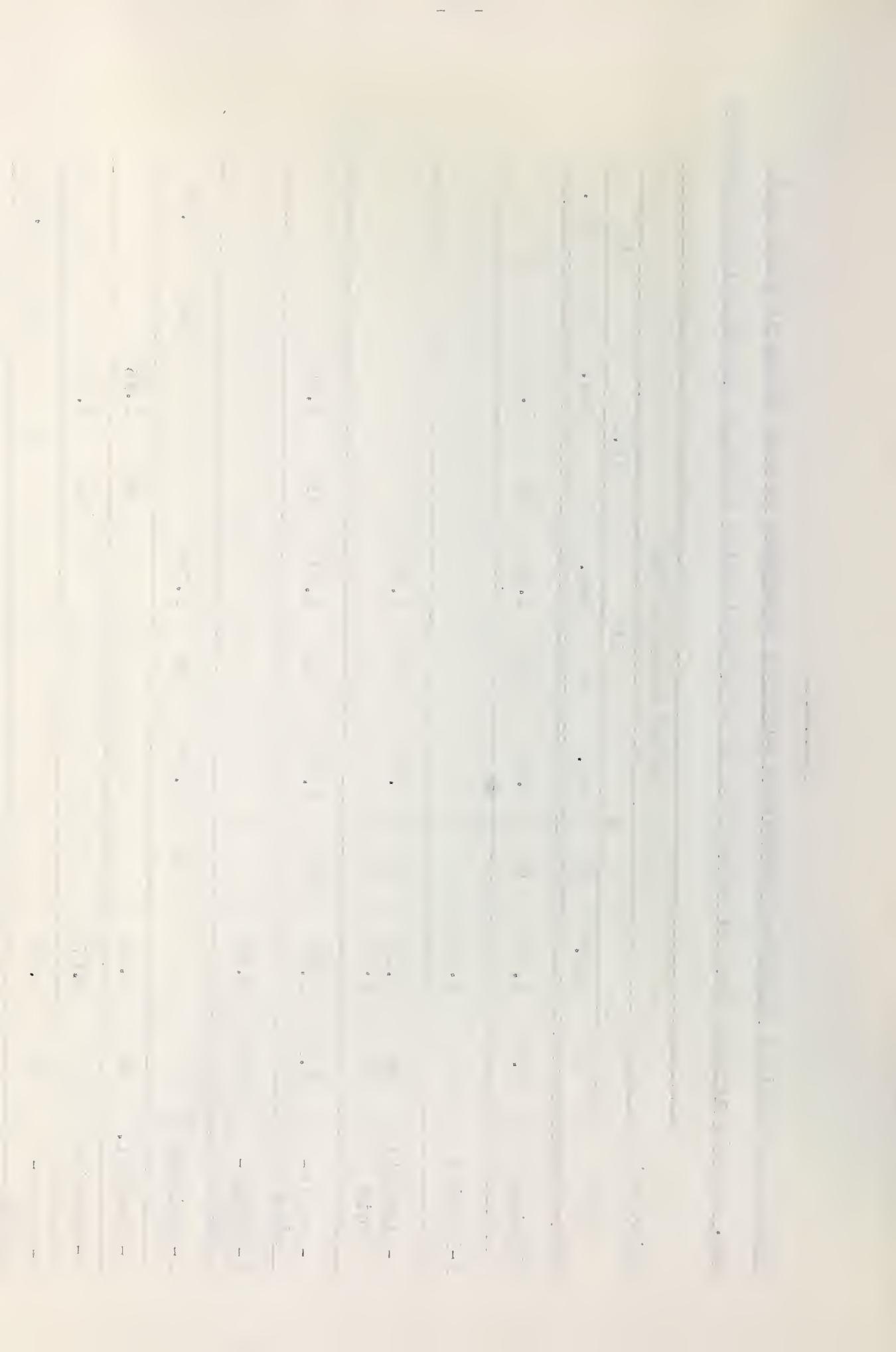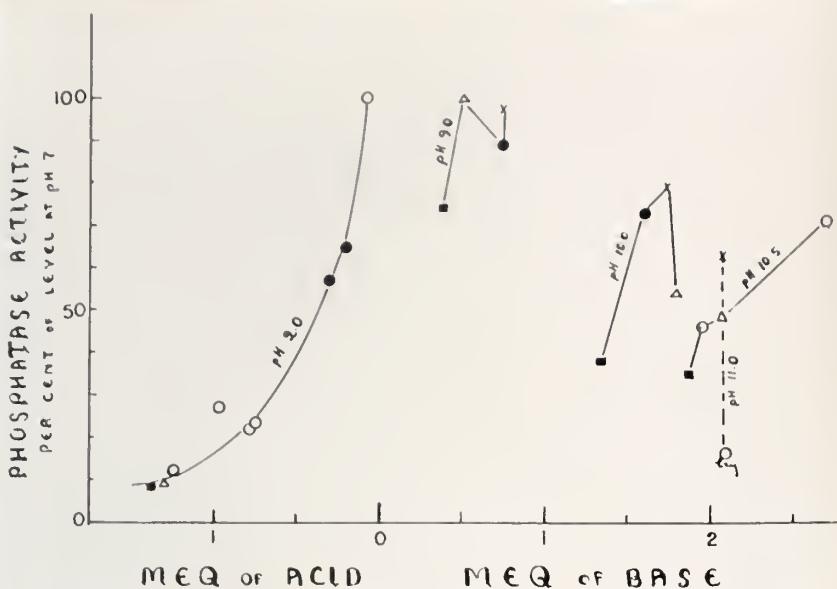
Figure 12a.
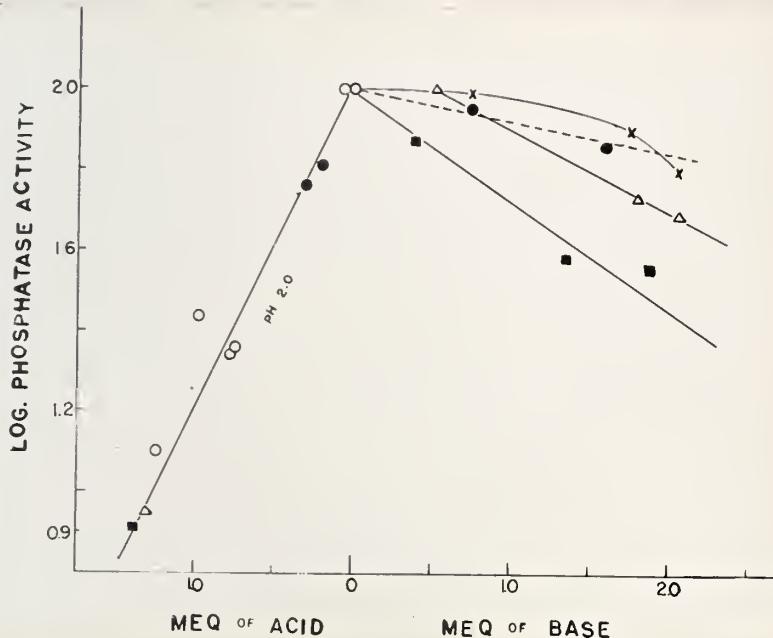
Figure 12b. Effect of force-feeding acid and alkaline solutions of various Amino Acids on the Intestinal Alkaline Phosphatase actimity.
(■)=glycine,(△)=methionine,(●)=glutamic acid,
(×)=arginine,(○)=(from left to right): leucine,
phenylalanine,lysine,tryptophan,tyrosine on pH 2.0
line,phenylalanine,tryptophan and tyrosine on the
alkaline side.

amino acids at pH 2.0 appears to depend only on the amount
of the hydrochloric acid used to adjust the pH to 2.0. The
contribution of the amino acids to this phenomenon is only
indirect. Since each one of them has a different buffering
capacity, varying amounts of hydrochloric acid were required
in each case to bring the pH to 2.0, and consequently vary-
ing degrees of phosphatase inhibition were produced each
time, when these solutions were fed to rats.

When the number of milliequivalents of base was
plotted against residual phosphatase activity (expressed
again as per cent of the value obtained after force-
-feeding a pH 7 solution of the same amino acid) there was
no correlation between the two, if they were compared accord-
ing to the pH of the amino acid solutions which were force-
-fed to the animals (see Figure 12a). However, a reasonably
straight line could result, if the comparison were made accord-
ing to the individual amino acid force-fed after adjustment
to various alkaline pH values. The logarithms instead of the
percentages of the residual phosphatase activities were plotted
in Figure 12b. A straight line was again obtained except in
the case of arginine. The points are very few though (only
three for each individual amino acid) and in most cases they
represent the average of the activities of merely two rats.
It is not possible therefore to know definitely whether a
linear or an exponential relationship exists between
amount of base and phosphatase activity. In any case
it seems justified to conclude that the inhibition exerted by

alkaline solutions of amino acids is dependent on the amount of base force-fed each time as well as on the nature of the individual amino acid. Thus glycine solutions always produced a greater degree of inhibition than solutions of methionine, glutamic acid, or arginine of the same pH. However, it should be noted that the amounts of base required to adjust the solutions of the above amino acids to certain alkaline pH values, were always less in the case of glycine. The degree of inhibition decreased in the order listed. Of the rest of the amino acids tested tryptophan seemed to be the most toxic of all to the enzyme, while tyrosine was the least inhibitory.

It is not difficult to understand the dependence of the degree of inhibition of the enzyme on the amount of base or acid which was force-fed each time along with the amino acid rather than on the pH of duodenal fluid. The living animal is not a test tube i.e. it does not represent a stationary system. Amounts of acid or base are continuously absorbed and during this process come in contact with the enzyme. Consequently they can affect the ionization of its active groups or denature the enzyme protein. The larger the amount of ~~molecules of~~ acid or base absorbed the greater the effect on the enzyme.

The difference in inhibition of the intestinal alkaline phosphatase activity manifested by the various amino acids tested in alkaline solutions is not so easy to explain. Differences in the ability of these amino acids to combine with bases and consequently to reduce the amount of free NaOH absorbed

may be responsible. Glutamic acid as a dicarboxylic acid
can neutralize more NaOH than methionine or glycine.
Methionine could also neutralize larger amounts of base .
than glycine because the pK_2 value of methionine is lower (9.21)
than the pK_2 of glycine (9.78).

Arginine must be considered separately. The pH of a
0.76M solution of this amino acid is 11.2-11.4. As a result
of this HCl acid was used to adjust the pH to the desired
level. If the number of meq. of acid added were plotted vs. log
residual phosphatase activity the same type of curve as that
represented in Figure 12b would be obtained. The only
difference would be that now it would be moved over to the
acid side of the figure. It would consequently represent the
reverse of the relationship between number of meq. of acid and phosphatase
activity noted with the other amino acids whose pH was adjusted
to 2.0 i.e. phosphatase activity increases with increasing
amounts of acid. This may be explained by the assumption
that the effect of acid on the ionizable groups of the enzyme
active site(s) is counteracted by an opposite action of
arginine.

No relationship was found to exist between degree of
hemorrhages in the gastrointestinal tract and phosphatase
activity. Generally the anatomical damage was more pronounced
among the animals which were force-fed alkaline solutions,
while the inhibitory effect on phosphatase was greater in the
animals fed the acid solutions.

SUMMARY

1. Amino acid solutions of pH 2.0 were found to depress
   intestinal alkaline phosphatase activity, when they were
   force-fed to starved albino rats repeatedly. An
   exponential relationship was discovered to exist between
   meq's. of acid added to the solution which was fed to
   the animals and residual phosphatase activity. The
   regression equation calculated from the experimental data
   was: log percent phosphatase activity = 0.764x + 0.814.
   The inhibition was independent of the nature of the amino acid.

2. Solutions of amino acids of pH 9.0, 10.0, 10.5, and 11.0
   were also found to be inhibitory, but ~~in~~ the degree of
   inhibition was dependent on the nature of the amino acid
   tested as well as on the meq. of base added to the solution.

3. Inactivation of intestinal alkaline phosphatase activity
   was also observed after a single force-feeding of pH 2.0
   solutions of methionine to fasted as well as to normal
   animals, provided that there were not large amounts of food
   present in the stomachs of the rats at the time of force-
   feeding. Food seemed to counteract this inhibitory effect
   of hydrogen ions.
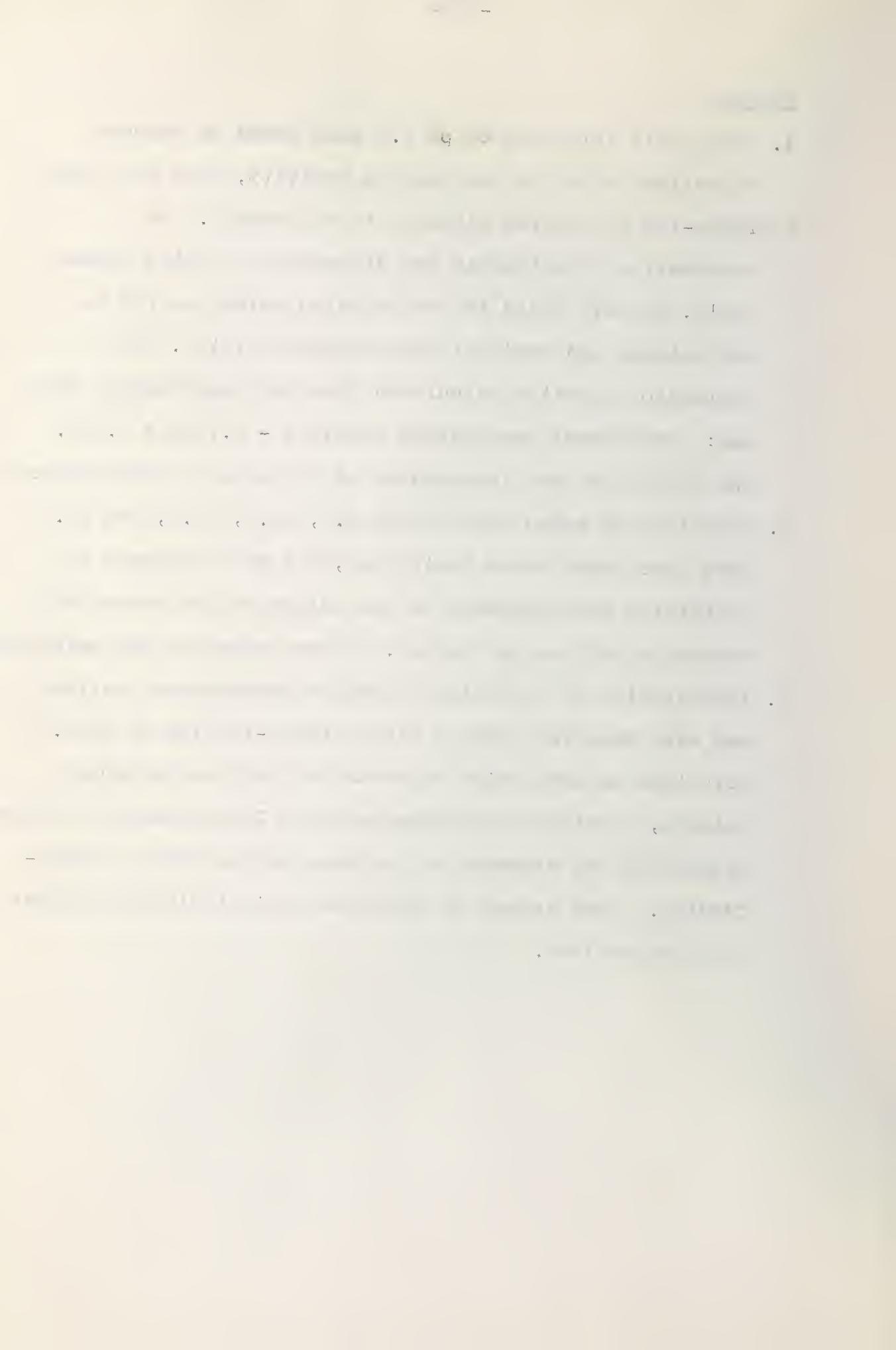
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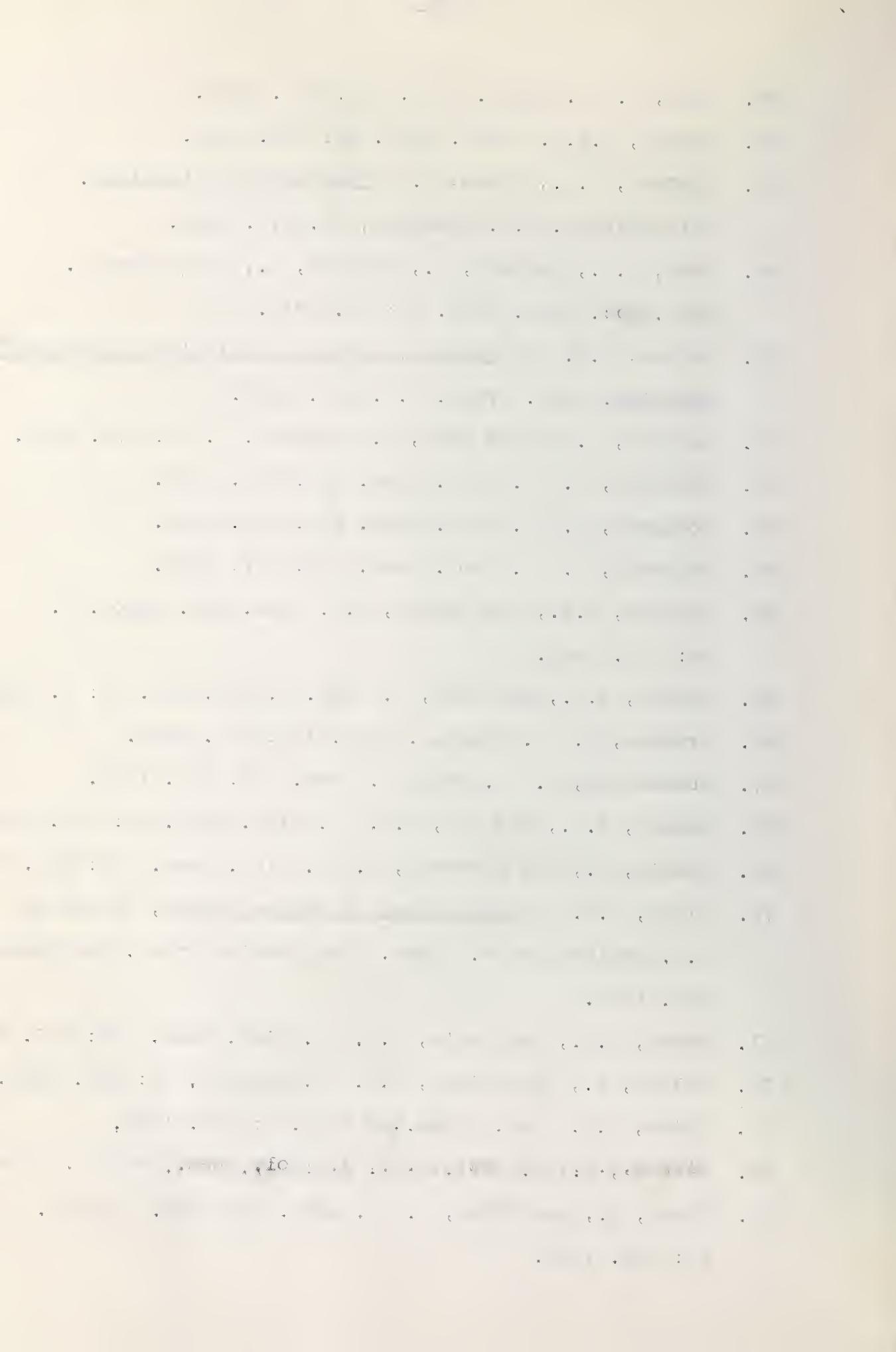
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